The Generation of MHC Class I Restricted Islet Antigen Specific Regulatory T Cells for the Treatment of Type 1 Diabetes

Nickolay, Lauren Emma

Awarding institution:
King's College London

The Generation of MHC Class I Restricted Islet Antigen Specific Regulatory T Cells for the Treatment of Type 1 Diabetes

by

Lauren Emma Nickolay

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Peter Gorer Department of Immunobiology
Kings College London
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Abstract

Type I diabetes (T1D) is an autoimmune disease in which the insulin producing β cells of the pancreas are selectively targeted for destruction by autoimmune effector T cells. The aberrant effects of these effector T cells may in part be due to a dysfunction in the regulatory T cell (T_{REG}) compartment and currently there is great interest in developing methods to strengthen the immunoregulation of patients with T1D. A potential way to boost immunoregulation in these patients would be the use of adoptive T_{REG} therapy whereby expanded polyclonal T_{REG} are transferred into patients. Although this treatment in mouse models of disease has shown promise it has been found that antigen specific T_{REG} cells are much more efficacious of preventing disease, and can even reverse disease.

The translation of these murine experiments into the human setting is however complex, since the generation of large numbers of antigen specific T_{REG} from human patients is currently a major hurdle. One way to remove this barrier is to utilise lentiviral gene transfer technology, which can allow for the transfer of antigen specific T cell receptor (TCR) genes into a desired cell population. Specifically for T1D, it is hypothesised that T_{REG} engineered to express a MHC Class I restricted (MHCI) TCR, although unconventional, would selectively function at the site of inflammation i.e. within the islets. This project, therefore, aims to generate MHCI islet antigen specific T_{REG} with the hypothesis that these would confer islet antigen specific suppression.

To test this hypothesis we engineered human $CD4^+$ T_{REG} to express two MHCI islet antigen specific TCRs whilst using a third high affinity pathogenic MHCI TCR as a control. As others have shown, we demonstrate that the control TCR was effectively able to re-direct the antigen specificity of T_{REG} cells through signalling and function. However, we discovered that transfer of autoimmune MHCI TCRs were unable to yield the same results as the control TCR due in part to their natural low affinity for antigen.

To circumvent this, we engineered $CD4^+$ T_{REG} to express an MHCI autoimmune TCR along with the CD8αβ co-receptor or a CD8αβ high affinity

variant. Using this system, human T_{REG} could be successfully re-directed towards an islet specific peptide and exhibit antigen specific suppression. Thus, this study is the first of its kind to use an autoimmune disease relevant, MHCI TCR to successfully re-direct the Ag specificity of T_{REG} cells.

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Abbreviations

7-AAD	7-Aminoactinomycin D
AAV	Adeno-Associated Virus
ADA	Adenosine deaminase
Ag	Antigen
Aire	Autoimmune Regulator Gene
Amp	Ampicillin
APC	Antigen Presenting Cell
APS-1	Autoimmune polyendocrinopathy syndrome type 1
C	Constant
CAR	Chimeric Antigen Receptor
CDR	Complementary determining regions
CMV	Cytomegalovirus
cPPT	Central polypurine tract
CTL	Cytotoxic T Lymphocytes
CTLA-4	Cytotoxic T-lymphocyte-associated Protein- 4
D	Diversity
DC	Dendritic cell
DMSO	Dimethyl sulphoxide
EF1-α	Elongation Factor 1-α
ELISA	Enzyme-linked immunosorbant assay
FACS	Fluorescent Activated Cell Sorting
FCS	Foetal calf serum
GMP	Good manufacturing practice
GWAS	Genome Wide Association Studies
HA	Haemagglutinin antigen
HEK 293T	Human embryonic kidney epithelial cells
HEL	Hen egg lysosome
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
idd	Insulin dependent diabetes
IFN	Interferon
IL	Interleukin
IRES	Internal ribosome entry site
ITAM	Immunoreceptor tyrosine-associated based activation motifs
J	Joining
LAT	Linker for activation of T cells
LTR	Long Terminal Repeat
LV	Lentivirus
MFI	Mean Fluorescence Intensity

MHC	Major Histocompatability Complex
MHCI	Major Histocompatability Complex Class I
MHCII	Major Histocompatability Complex Class II
MoMLV	Moloney Murine Leukaemia Virus
NK	Natural Killer
NOD	Non-obese diabetic
NPPE	Naturally Processed and Presented Epitope
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PHA	Phytoheamagluttinin
PIC	Pre integration complex
PPI	Preproinsulin
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RAPA	Rapamycin
RIP	Rat insulin promoter
RRE	Rev responsive element
RSV	Rous Sarcoma Virus
ScFv	Single chain variable fragment
SCID	Severe Combined Immunodeficiency
SIN	Self inactivating
SNP	Single nucleotide polymorphism
T1D	Type 1 Diabetes
TCR	T cell receptor
T_{EFF}	Effector T Cell
TGF	Transforming Growth Factor
T_H	T helper cell
T_{REG}	Regulatory T Cell
TSDR	T_{REG} Specific demethylation region
UCB	Umbilical Cord Blood
V	Variable
VNTR	Variable number of tandem repeats
VSV-G	Vesicular stomatitis virus protein G
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
WT	Wild type
Zap70	ζ-chain associated protein kinase of 70kDa
β_2M	β_2 Microglobulin

1. Introduction

1.1. Adaptive Immunity – A Double Edged Sword

The immune system is a complex collection of cells and molecules that mediate the protection of the body from harmful and infectious pathogens. For efficient protection, the immune system has two arms of defence – the innate immune system providing an immediate response to pathogens and the adaptive immune system that although taking longer to respond, provides exquisite specificity and memory to efficiently eliminate infections. In the absence of a fully functioning adaptive immune system, such as in cases of severe combined immunodeficiency (SCID) or acquired immunodeficiency syndrome (AIDS), the body is overwhelmed by opportunistic pathogens, which would normally have been cleared. The specificity required by the adaptive immune system is provided by T and B-lymphocytes, which through the expression of highly specialised antigen (Ag) specific receptors enable the body to detect virtually every potential invading pathogen.

There are not enough genes within the whole human genome (estimated 20,000-25,000 protein coding genes) to fully encode all individual Ag receptor specificities that are required to protect the body. Therefore, to achieve this diversity, the Ag specific receptor genes of lymphocytes undergo random recombination events (Tonegawa, 1983). The recombination events occur within variable (V) regions of each of the two chains that comprise an Ag specific receptor. Each variable region is composed of two or three gene segments and each of these gene segments is present in multiple copies within germline DNA (Early et al., 1980). Thus, gene recombination refers to the random joining of single copies from each set of gene segments to form a rearranged V region. In a second random process, two receptor chains are brought together to form a functional Ag specific receptor adding to the diversity within the repertoire of Ag specificities. Although this random nature is key to the development of a vast repertoire of Ag specific receptors

capable of recognising and destroying all pathogens, it also provides a platform for the development of receptors that recognise self-Ags and have the potential to target healthy tissue. This in short describes the doubled edged sword of adaptive immunity and these self-reactive lymphocytes are widely reported as the perpetrators of many autoimmune diseases including type 1 diabetes (T1D), multiple sclerosis (MS) and rheumatoid arthritis (RA) (Firestein, 2003; Roep and Peakman, 2011; Steinman, 1996).

T lymphocytes, or T cells, derive from the common lymphoid progenitor and migrate to the thymus from the foetal liver and adult bone marrow for development. During development, they gain expression of an Ag specific receptor known as a T cell receptor (TCR). Each TCR is composed of two chains, TCRα and TCRβ, the structural composition of which defines the specificity of a TCR. During T cell development, the TCRα and TCRβ chains undergo V(D)J recombination, which are the somatic recombination events that combine variable (V), diversity (D) and joining (J) gene segments to form functional TCR chains. This process generates diversity in two ways 1) by combining one each of a multitude of V D and J segments and 2) introducing random nucleic acid insertions or deletions at the joints between gene segments (Davis and Bjorkman, 1988). In fact, it is estimated that at any one time T cells with $25x10^6$ unique specificities can be identified in human peripheral blood (Arstila et al., 1999). Importantly, TCRs only recognise Ag processed into smaller sized peptides and presented in the context of major histocompatability (MHC) cell surface molecules (Babbitt et al., 1985). There are two types of MHC molecules known as MHC class I (MHCI) and MHC class II (MHCII) molecules. T cells that express TCRs that recognise Ag in the context of MHCI express the CD8 co-receptor and those that recognise Ag in complex with MHCII express CD4.

As mentioned, the generation of a diverse TCR repertoire is vital to the combatant of complex pathogens but has the additional downfall of enabling the development

of self-reactive T cells. Therefore, a number of mechanisms exist to prevent the development or action of T cells with reactivity to self. During T cell development, two checkpoints are in place to maintain central tolerance. Firstly, only T cells that possess TCRs that have a low level interaction with self-peptide MHC (pMHC) complexes in the thymus are positively selected for T cell development. Secondly, during a process known as negative selection, cells that react too strongly with self-pMHC are deleted to prevent the release of autoreactive T cells. Alternatively, cells that react to self-pMHC can be converted into regulatory T (T_{REG}) cells that function in the periphery to inhibit self-reactive T cells. As negative selection is not absolute, potentially due to an absence or low level expression of self Ag in the thymus, autoreactive T cells can exit into the periphery. To prevent the activations of these autoreactive T cells a number of mechanisms, collectively known as peripheral tolerance, exist. These mechanisms include anergy, a functional unresponsiveness in cells that encounter Ag in the absence of co-stimulation, clonal deletion when apoptosis is induced in chronically stimulated T cells and the action of T_{REG} cells (Xing and Hogquist, 2012).

Despite these complex mechanisms of central and peripheral tolerance, adaptive immune responses are elicited against self-Ag and tissues and autoimmunity occurs in ~5% of individuals in developed countries (Jacobson et al., 1997). Both genetic and environmental factors can play a role in the breakdown of tolerance and T1D is a prototypical autoimmune disease in which the adaptive immune response elicits a sustained and detrimental attack on self-tissues (Bluestone et al., 2010).

1.2. Type 1 Diabetes – An Autoimmune Disease

1.2.1. Aetiology of Type 1 Diabetes

T1D is the result of an immune cell mediated destruction of the insulin producing β cells within the pancreas (Eisenbarth, 1986). A decline in insulin production can render patients with an inability to control their blood glucose levels, which can

have severe acute complications as well as secondary complications such as heart disease and kidney failure (Vauzelle-Kervroedan et al., 1999). At the turn of the 20th century, the diagnosis of T1D was relatively rare and with no treatments available, the disease was fatal, with an average life expectancy after diagnosis of only 6.1 years (Joslin, 1950). With the remarkable discovery of insulin by Drs Banting and Best in 1922 came the ability for physicians to treat T1D with insulin replacement therapy leading to an increase in patient survival (Banting and Best, 1922). The last 90 years have seen many advances in T1D management, including recombinant insulin, home glucose testing and more recently the use of insulin pumps, which allow the provision of insulin through a catheter inserted under the skin as opposed to delivery by patient administered injections. Currently, trials are underway using "artificial pancreata" that combine glucose monitoring systems, insulin pumps and control algorithms to automate glycemic management and initial results have shown improved glycemic control compared to a canonical insulin pump (Russell et al., 2014). Overall, insulin replacement therapy is still the only means to treat the symptoms of T1D and the improved management and diagnosis of disease has led to a further increase in the life expectancy of patients. However, a study in 2001 of a T1D cohort found that this group of patients still had a two and a half times greater mortality rate than a healthy control cohort (Brown et al., 2001). It is well documented that the incidence of T1D is increasing worldwide (Gale, 2002) and it has been predicted that T1D development in children under the age of five will double by 2020 (Patterson et al., 2009). With the incidence of T1D increasing at an alarming rate, the reality of a social and economic burden of this disease is becoming clear. It is therefore becoming imperative to engineer ways to treat or cure the underlying causes of T1D as opposed to the current methods of treating the symptoms.

The late George Eisenbarth first proposed a model of T1D pathogenesis in 1986, which described a chronic autoimmune disease that could be divided into distinct stages (Eisenbarth, 1986). These stages included a genetic susceptibility which, when followed by a triggering event, could lead to the autoimmune destruction of β

cells perpetrated by autoreactive T cells. Eisenbarth's model has been adapted over the years to incorporate new data garnered from both human studies and studies in the non-obese diabetic (NOD) mouse model of T1D (Figure 1-1).

Despite nearly 30 years of study since the proposal of this model, the defining trigger that causes the development of T1D has yet to be discovered. It has however been postulated that there is a strong link between genetic predisposition and environmental factors in T1D development (Bluestone et al., 2010).

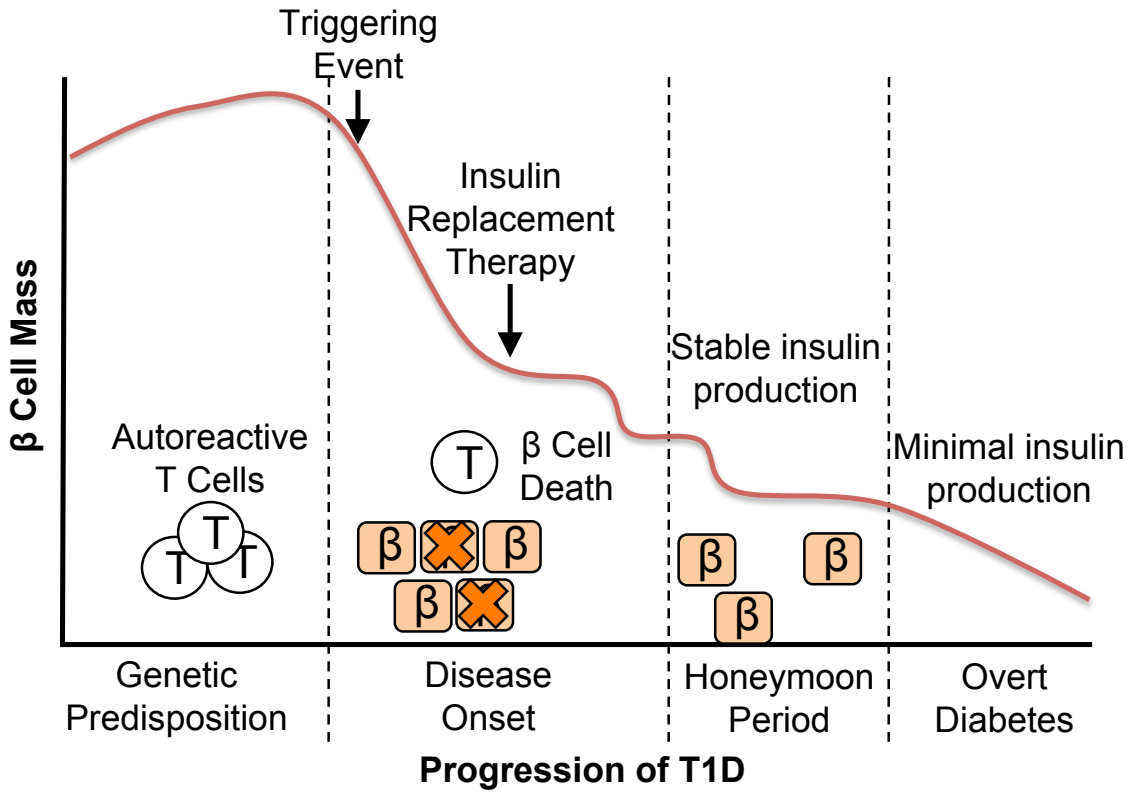

Figure 1-1. Model for T1D Disease Progression. T1D can affect people who have a genetic predisposition to disease in addition to circulating autoreactive T cells. At disease onset, an unknown event triggers disease, which culminates in autoreactive T cell mediate destruction of the insulin producing β cells. Symptomatic patients will be treated with insulin replacement therapy that leads to a metabolic stabilisation known as the "honeymoon period". However, increased β cell death can lead to further reduction in insulin production that is increasingly difficult to control with insulin replacement therapy and overt T1D prevails.

1.2.2. The NOD Mouse Model of T1D

The NOD mouse is an inbred strain that spontaneously develops type 1 autoimmune diabetes (Makino et al., 1980). Whilst not a perfect model of disease, the NOD mouse shares many of the features of human T1D, including pathogenesis, genetics and target auto-Ags. This model is therefore used to study many aspects of autoimmune diabetes, in particular it is used as a model to test potential therapies that may be used to treat human disease.

1.2.3. Factors leading to the development of T1D

As previously mentioned, T1D development involves a complex interplay between genetic and environmental factors. A strong rationale for the involvement of both these factors is evidenced by only a 50% concordance rate of T1D between monozygotic twins (Redondo et al., 2008). Additional evidence of a role for genetics comes from genome wide association studies (GWAS), which have identified over 40 “at risk” genetic loci for T1D (Barrett et al., 2009).

In human disease, the strongest genetic associations with T1D development are within the Human Leukocyte Antigen (HLA) genes that encode MHC molecules. Certain HLA Class I and Class II alleles have been found to have a strong genetic predisposition for T1D, whereas others have been found to have a protective association (Howson et al., 2009; Nejentsev et al., 2007). In particular, specific alleles within the MHCII molecules DRB1, DQA1 and DQB1 loci are strongly associated with T1D development (Cucca et al., 1993). MHC molecules possess a peptide binding groove that is selective for the length and shape of the peptide with which it can complex and subsequently present to T cells. As mentioned, self-pMHC complexes are vital to the processes of positive and negative selection of T cells in the thymus; therefore, the MHC molecules that an individual possesses can dictate and shape the TCR repertoire. Another gene polymorphism with a high genetic association with T1D is allelic variation within the promoter of the insulin (*INS)* gene. Variation at the Insulin gene’s ‘variable number of tandem repeats’ (VNTR) locus can predispose individuals to T1D by altering the level of insulin that

is present in the thymus. It has been shown that individuals that are homozygous for the susceptible class I *INS* allele have lower insulin mRNA expression in the thymus compared to individuals homozygous for the protective class III *INS* allele (Pugliese et al., 1997; Vafiadis et al., 1997). The reduced expression of insulin in the thymus could lead to a reduction in negative selection of insulin reactive T cells. Furthermore, it could also lead to a reduction in the thymic generation of T_{REG} cells specific for insulin peptides.

Interestingly, several genes associated with T1D susceptibility are thought to play a key role in T_{REG} cell development or function, (Todd, 2010) (Figure 1-2) for example, there are three T1D associated polymorphisms within the Interleukin-2 receptor α (*IL2RA*) and one within the *IL2* gene. IL-2Rα, also known as CD25, is a component of the high affinity IL-2R complex and is constitutively expressed on the surface of T_{REG} cells (Sakaguchi et al., 1995). A vital role for IL-2Rα and IL-2 protein has been shown in the generation and function of T_{REG} cells, which includes the maintenance of peripheral tolerance to self Ags (Bayer et al., 2005; Burchill et al., 2007; Malek and Bayer, 2004). Furthermore, defects in the T_{REG} cell compartment have been found in patients with T1D, which may be linked to gene polymorphisms within the *IL2RA* and *IL2 genes* (Brusko et al., 2005; Lawson et al., 2008; Lindley et al., 2005).

Another polymorphism associated with T1D susceptibility is found within the Cytotoxic T-lymphocyte associated protein-4 (CTLA-4) gene, which encodes CTLA-4 an important regulator of T cell activation that is constitutively expressed by T_{REG} cells (Manzotti et al., 2002; Ueda et al., 2003). This polymorphism has been suggested to affect T_{REG} cell function. The protein tyrosine phosphatase, non-receptor type 22 (PTPN22) is also involved in regulating T cell activation and a single nucleotide polymorphism (SNP) within the *PTPN22* gene is associated with T1D and many other autoimmune diseases. It has been suggested that this SNP increases the TCR signalling threshold, which has a negative effect of T_{REG} cell thymic development (Maine et al., 2012). Each of these genes described play a

fundamental role in immune regulation suggesting that disease susceptibility is underpinned by a defective regulation of immune responses.

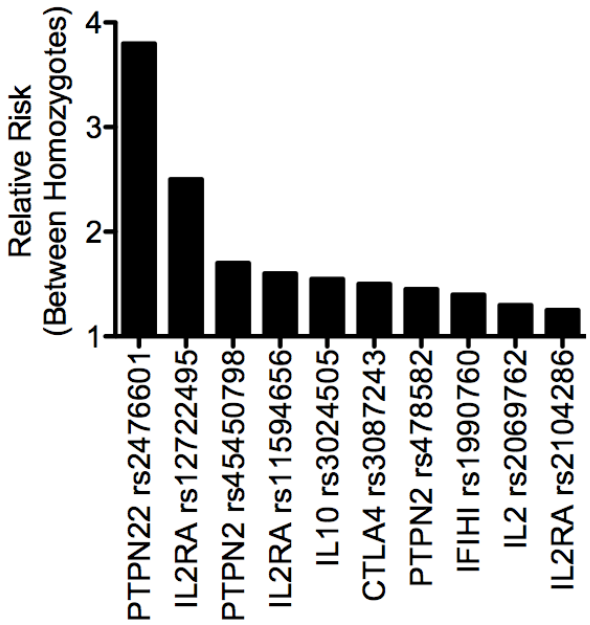

Figure 1-2 T1D associated gene polymorphisms involved in immune regulation. The relative risk of several T1D associated gene polymorphisms with a role in immune regulation is shown. Data adapted from John Todd, Etiology of T1D, Immunity, 2010 (Todd, 2010).

These observations in humans are closely mirrored in the NOD mouse. The most dominant genetic loci associated with disease in the NOD mouse model is also within the genes that encode MHC molecules. In fact, the NOD mouse possess a unique MHC haplotype, known as I-A^{g7}, which is essential for diabetes susceptibility (Wicker et al., 1995). Interestingly, T1D susceptibility in humans is linked a missing aspartic acid residue within the β chain of HLA-DQ alleles and this residue is similarly lacking in I-A^{g7} (Acha-Orbea and McDevitt, 1987; Todd et al., 1987). Additionally, a number of other disease susceptibility loci have been identified in the NOD mouse and are termed insulin dependent diabetes (*idd)* loci. A number of genes with a role in immune regulation have been mapped to these regions including polymorphisms in the *CTLA4*, and *IL2* genes.

It is widely recognised that genetic susceptibility alone does not correlate with T1D progression. Even in the disease susceptible NOD mouse strain, diabetes spontaneously develops in only 60-80% female and 20-30% of male mice. Interestingly, NOD mice that are stored in germ-free conditions have a higher incidence of T1D and exposure to certain microbes is found to protect these mice from T1D development (Singh and Rabinovitch, 1993). A similar phenomenon has been identified in humans and is evidenced by a reduced T1D incidence in societies where there is a high risk of infectious disease (Bach, 2002). This theory is known as the hygiene hypothesis. Additionally, there is strong evidence that T1D is triggered in humans by enteroviral infection. Studies have shown that Coxsackie virus B4 can be isolated from the pancreata of newly diagnosed patients (Yin et al., 2002; Yoon et al., 1979). Furthermore, studies have suggested that enterovirus possess tropism for human β cells and that infection with enterovirus can exacerbate the development of diabetes in the NOD mouse model (Dotta et al., 2007; Serreze et al., 2000). Further environmental factors that have been identified in T1D include Ags commonly found in dietary products and level of vitamin D exposure (Norris et al., 2007; Virtanen et al., 2006; Weintrob et al., 2001).

1.2.4. Immune Mediated Pathogenesis of T1D

Studies in the NOD mouse model and in patients with T1D have provided strong evidence that autoreactive T cells mediate the destruction of insulin producing β cells. However, there is evidence that cells of the innate immune system, such as natural killer (NK) cells, macrophages and dendritic cells (DCs), mediate the initial islet inflammation. Macrophages and dendritic cells have been identified in the pre-diabetic lesion of NOD mice prior to the recruitment of autoreactive T cells (Dahlen et al., 1998). It is thought that low-grade inflammation of the islets can recruit APCs, which can pick up and process β cell Ags. These APCs can then migrate to the pancreatic draining lymph node where β cell Ags are presented to islet autoreactive T cells, which are subsequently activated and endowed with the capacity to migrate to the islets. The presentation of β cell Ags to islet autoreactive

T cells in the pancreatic draining lymph node has been demonstrated in the NOD mouse (Turley et al., 2003). B cells are also thought to play a major role in the initial presentation of islet Ags to autoreactive T cells and depletion of B cells in pre-diabetic NOD mice has been shown to prevent disease progression (Xiu et al., 2008).

A major role of autoreactive T cells in T1D is evident by the identification of insulin reactive $CD4^+$ and $CD8^+$ T cells in the circulation of patients with T1D (Kent et al., 2005; Skowera et al., 2008) and in the NOD mouse (DiLorenzo and Serreze, 2005). In particular, $CD8^+$ T cells have been shown to be the key mediators of β cell death; this was first evident following observations that MHCI was hyper expressed in the islets of patients with T1D (Foulis et al., 1987; Itoh et al., 1993). An increase in MHCI molecules in complex with β cell Ags have the potential to enhance the activation of low affinity autoreactive CD8 T cells. Further evidence has been identified using in-depth immunohistochemistry staining of pancreatic samples from patients with T1D. In these sections, $CD8^+$ T cells were found to be the most abundant cell infiltrate, followed by $CD68^+$ Macrophages and to a lesser extent, $CD4^+$ T cells (Willcox et al., 2009). Furthermore, an elegant study utilised *in situ* MHCI tetramer staining of pancreatic sections from T1D patients and detected autoreactive $CD8^+$ T cells within the islets (Coppieters et al., 2012).

Autoreactive $CD4^+$ T cells, although not as abundant in insulitis as $CD8^+$ T cells have also been shown to contribute to T1D development and pathogenesis in humans. $CD4^+$ T cells from T1D patients show increased proliferation to β cell Ags in comparison to HLA matched non-diabetic controls (Peakman et al., 1999; Roep et al., 1995). Additionally, there is strong evidence that human β cells are highly susceptible to apoptosis by cytokines secreted by $CD4^+$ T helper 1 (T_H1) cells such as IFN-γ and TNF-α in combination with macrophage secreted IL1-β (Cnop et al., 2005). In particular, IFN-γ secreting cells in response to MHCII restricted islet Ags have been identified in patients with T1D (Arif et al., 2004). Moreover, a role for T helper 17 (T_H17) cells has been described with the identification of IL-17 secreting

$CD4^+$ T cells in the peripheral blood of newly diagnosed patients (Arif et al., 2011; Honkanen et al., 2010; Marwaha et al., 2010). Furthermore, IL-17 has been found to contribute to cytokine mediated β cell apoptosis (Arif et al., 2011), which suggests that a primed pro-inflammatory response mediated by autoreactive $CD4^+$ T cells contributes to β cell death and T1D progression.

In combination with genetic evidence, it is clear that autoreactive T cells that escape negative selection in the thymus mediate T1D (Roep and Peakman, 2011). However, multiple studies have identified the presence of autoreactive T cells in individuals with no evidence of T1D suggesting that other factors mediate the pathogenesis seen in T1D patients and in the NOD mouse (Arif et al., 2004; Danke et al., 2005). As mentioned, a number of genetic loci that predispose to T1D development are involved in the complex process of immune regulation and defects in this process have been widely studied in the context of T1D.

1.2.5. Impaired Immune Regulation in T1D

Peripheral tolerance consists of a number of mechanisms that the immune system has developed to prevent the activation of T cells to self- or non-harmful Ags such as commensal bacterial or dietary Ags (Xing and Hogquist, 2012) and there is overwhelming evidence that T1D occurs in part due to a failure in immune regulation (Roep and Tree, 2014). A major mediator of immune regulation is the specialised T cell subset known as T_{REG} cells. A number of T_{REG} cell subsets have been characterised; however, the most abundant and well-characterised subset are defined by co-expression of CD4 and CD25. These cells stably express the transcription factor FoxP3, which is the master regulator of $CD4^+CD25^+$ T_{REG} cells and can develop either in the thymus, or in the periphery where naïve $CD4^+$ T cells can take on a regulatory phenotype. Over the past decade there has been an often confusing nomenclature used to describe different populations of cells with regulatory function (Abbas et al., 2013). In this thesis, I will use the term T_{REG} cell to refer to $CD4^+CD25^+FoxP3^+$ regulatory T cells isolated from or present in the periphery of mouse and man.

$CD4^+$ T cells that possess a TCR with a relatively high affinity for self-peptide can be deleted in the thymus by negative selection or undergo a program of differentiation into a T_{REG} cell (Sgouroudis et al., 2008). These cells then migrate into the periphery where they can mediate peripheral tolerance using a variety of suppressive mechanisms. These mechanisms include: secretion of suppressive cytokines such as IL-10, IL-35 and TGF (transforming growth factor)-β, granzyme and perforin mediated killing of APCs and CTLA-4 expression (Collison et al., 2007; Gondek et al., 2005; Gregg et al., 2004; Puccetti and Grohmann, 2007; Rubtsov et al., 2008; Takahashi et al., 2000). CTLA-4 expression can inhibit co-stimulatory molecule expression of APCs and induce these cells to secrete metabolites with immune cell toxicity. Importantly, all the suppressive functions exhibited by T_{REG} cells reported to date require prior TCR stimulation, thus their highly self-reactive TCR repertoire is vital to preventing autoimmune diseases.

The strongest evidence that demonstrates the importance of an intact T_{REG} cell compartment comes from patients with the immune disorder immune dysregulation, polyendocrinopathy, enteropathy x-linked syndrome (IPEX). These patients have a loss of function mutation in their *FOXP3* gene and therefore have no T_{REG} cells and an overwhelming 80% of these patients develop T1D at a young age, irrespective of other genetic risk factors (Wildin et al., 2001). Furthermore, *scurfy* mice, which also have a defect in the *foxp3 gene*, develop severe and fatal autoimmune diseases including diabetes (Brunkow et al., 2001); this points to a clear role for T_{REG} cells in the prevention of T1D and although not as profound as observed in IPEX, a number of studies in mouse and man suggest that defects in the fitness and function of T_{REG} cells play a role in “normal” T1D pathogenesis.

In man, the majority of studies have identified that the function, but not the frequency of T_{REG} cells, differs between patients with T1D and healthy controls (Brusko et al., 2007; Glisic-Milosavljevic et al., 2007a; Glisic-Milosavljevic et al., 2007b; Lawson et al., 2008; Long et al., 2010). In classical *in vitro* co-culture suppression assays, T_{REG} cells from patients with T1D are less able to suppress

the proliferation of autologous effector T (T_{EFF}) cells in comparison to healthy controls. (Brusko et al., 2005; Lawson et al., 2008; Lindley et al., 2005). At least some of the observed reduction in T_{REG} cell function has been linked to an increased resistance to suppression of the T_{EFF} cells from patients with T1D (Lawson et al., 2008; Schneider et al., 2008). However, many studies have also identified additional defects within the T_{REG} cells of patients. Firstly, it has been observed that T_{REG} cells from patients are more prone to apoptosis (Glisic-Milosavljevic et al., 2007a; Glisic-Milosavljevic et al., 2007b). Moreover, in expanded populations of T_{REG} cells, patients with T1D have an increased proportion of IFN-γ secreting cells compared to healthy controls (McClymont et al., 2011). Furthermore, T_{REG} cells from patients with T1D have a reduced sensitivity to IL-2, which leads to a reduced maintenance in FoxP3 expression (Garg et al., 2012; Long et al., 2010). This reduced sensitivity to IL-2 may have an effect on T_{REG} cell fitness and persistence at the site of inflammation. Interestingly, staining of pancreatic sections from individuals with T1D revealed that the presence of FoxP3$^+$ T_{REG} cells in the islet cell infiltrate is uncommon (Willcox et al., 2009).

Several studies in the NOD mouse have suggested that although the frequency of T_{REG} cells is unaltered, the function and stability of T_{REG} cells is progressively lost as disease develops. It has been identified that within the inflamed islets of the NOD mouse there is an increase in the T_{EFF}:T_{REG} cell ratio (Tang et al., 2008). Additionally, genetic lineage tracing of FoxP3$^+$ T_{REG} cells in the NOD mouse revealed a proportion of inflammatory cytokine secreting "Ex-FoxP3$^+$" cells within the inflamed islets (Zhou et al., 2009). There is also evidence suggesting that reduced levels of IL-2 at the site of inflammation has a damaging effect on T_{REG} cell fitness and function. For example a mutation in the *idd3* allele found in the NOD mouse is associated with reduced levels of IL-2 production by activated T_{EFF} cells. Interestingly, the expression of a protective *idd3* allele in NOD mice reduced the incidence of diabetes and was concurrent with an increase in T_{REG} cell proliferation and suppressive function (Sgouroudis et al., 2008). Furthermore, although the frequency of T_{REG} cells is unaltered there is evidence that thymic

derived T_{REG} cells from the NOD mouse have a highly restricted TCR repertoire compared to other mouse strains (Ferreira et al., 2009). A reduced TCR diversity could compromise T_{REG} cells ability to prevent autoimmunity by restricting the range of self-peptide they can recognise. This defect was linked to a reduction in TCR signalling during negative selection in the thymus leading to a decrease in T_{REG} cell differentiation (Ferreira et al., 2014). Interestingly, another study in mice identified that a loss of the *PTPN22* gene, which encodes an inhibitor of TCR signalling, resulted in reduced T_{REG} thymic development (Maine et al., 2012). Coupled with the autoimmune disease associated SNP within the *PTPN22* gene, these data infer that TCR signalling can lead to a restricted T_{REG} cell repertoire that may be less well equipped to prevent autoimmunity.

It is still debatable whether reduced T_{REG} cell function in T1D patients is causative or a consequence of T1D (Roep and Tree, 2014). The T1D associated *IL2* polymorphism, rs12722495 has been shown to affect the phenotype of both T_{EFF} and T_{REG} cells and is proposed to contribute to the reduced T_{REG} cell function seen in T1D (Dendrou et al., 2009; Garg et al., 2012). Similarly to the *idd3* allele in the NOD mouse, this polymorphism reduced the amount of IL-2 produced by conventional T cells and can reduce the sensitivity of T_{REG} cells to this cytokine (Dendrou et al., 2009; Garg et al., 2012). Importantly, the study performed by *Garg et al* assessed the importance of this polymorphism on T_{REG} cell function in subjects with no evidence of disease. A second study by *Gilisic-Milosavljevic et al* identified that T_{REG} cell apoptosis was greater in patients with newly onset T1D and in subjects who are at a high risk of disease, as evidenced by the presence of two or three autoantibody specificities, compared to healthy control subjects (Glisic-Milosavljevic et al., 2007b). These studies suggest that the defects in T_{REG} cells are causative of T1D rather than a consequence of disease. However, there is a strong rationale that these defects, decreased sensitivity to IL-2 and increased apoptosis would be overcome by increasing T_{REG} cell number to boost overall suppression and prevent the occurrence or prohibit the progress of T1D.

Other T_{REG} subsets have been found to be impaired in patients with T1D. Studies have identified $CD4^+FoxP3^-$ cells that secrete high levels of the immunosuppressive cytokine IL-10 upon recognition of β cells Ags (Gregori et al., 2012; Petrich de Marquesini et al., 2010; Sanda et al., 2008). These cells have been shown to kill APCs pulsed with β cell Ags *in vitro* thereby preventing the function of effector T cells subsets (Tree et al., 2010). Interestingly, these IL-10 secreting cells are present in healthy controls and enriched in individuals who develop T1D at an older age versus those who are younger at disease onset suggesting they may play a role in disease prevention (Arif et al., 2004; Petrich de Marquesini et al., 2010).

$CD8^+$ T_{REG} cells have also more recently been identified as having a role in the suppression of T1D. Although these cells are less well characterised than $CD4^+$ T_{REG} cells, a study in 2010 showed that $CD8^+$ T cells specific for HLA-E bound to heat shock peptide hsp60sp could mediate self/non-self discrimination in the periphery (Jiang et al., 2010). This study also showed that $CD8^+$ T cells from patients with T1D had a defect in this self/non-self discrimination pathway; however, it could be rescued by vaccinations with autologous DCs loaded with hsp60sp. This study provided further evidence of an impaired immune regulation in patients with T1D.

1.2.6. Current Therapies to Strengthen Immune regulation

The overwhelming evidence that defective immune regulation contributes to T1D development has provided a strong rationale for the use of therapies designed to strengthen regulatory mechanisms (Figure 1-3). In the 1980's, it was recognised that non-specific immunosuppressive agents such as cyclosporine A could prevent T1D progression in newly diagnosed patients (Silverstein et al., 1988). Cyclosporine A functions by preventing IL-2 transcription and reduces the function of all T cells. However, the overall reduction in T cell activity causes large numbers of side effects that therefore limits its use in T1D, especially in the young. More recently, therapies aimed at preventing or reversing T1D have increased in their

immune specificity. These now include monoclonal antibodies (mAbs) that target specific immune cell subsets, islet Ag specific immunotherapy to promote Ag specific regulation and IL-2 therapy aimed at specifically increasing the T_{REG} cell compartment.

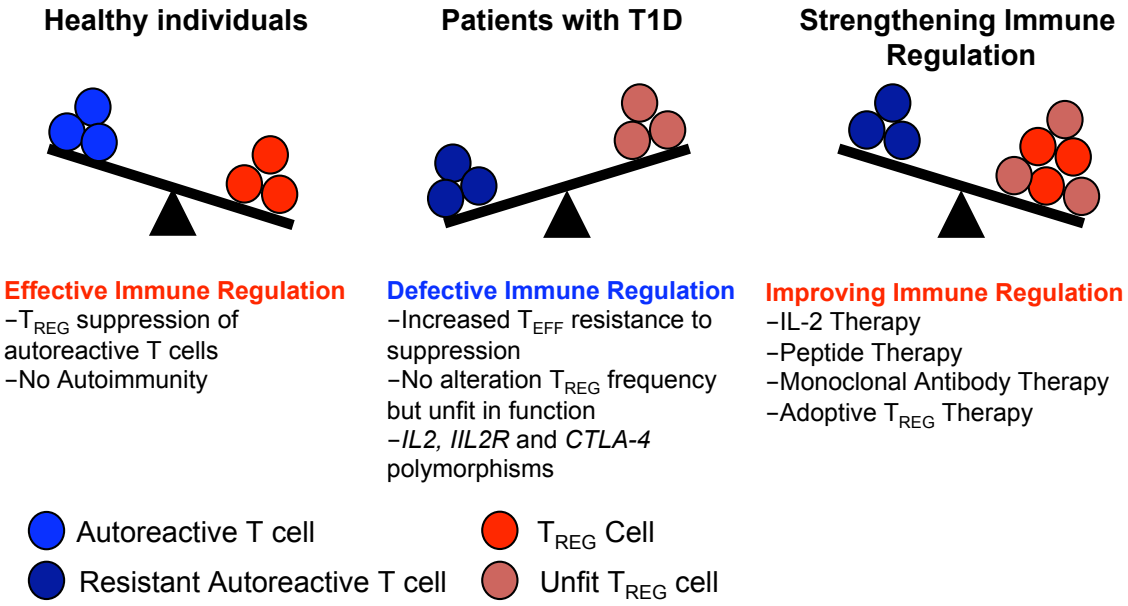

Figure 1-3 Re-addressing the balance of immune regulation and autoimmunity. In health, autoreactive T cells are kept in check by T_{REG} cells and autoimmunity does not occur. Patients with T1D have a genetic predisposition to reduced immune regulation with reduced fitness and suppressive function of T_{REG} cells. Prolonged inflammation ensures that T_{EFF} cells from T1D patients are more resistant to suppression, which also contributes to T1D progression. Therefore, therapies that are aimed to readdress the balance of immune regulation are being investigated to treat patients with T1D. The majority of these therapies are aimed at increasing T_{REG} cell number and fitness with a view of overcoming the dominant autoimmune response.

The use of αCD3 mAbs (teplizumab and otelixizumab) has been investigated as an immunotherapy in the treatment of T1D. In the NOD mouse, αCD3 treatment has been shown to prevent diabetes development in pre-diabetic mice and reverse disease in mice with overt diabetes (Chatenoud et al., 1994). It has been suggested that αCD3 mAbs can induce tolerance via a TGF-β mediated induction of T_{REG} cells (Belghith et al., 2003).

Six phase II or III clinical studies have been completed using teplizumab to treat patients with recent onset T1D (Daifotis et al., 2013). These trials showed efficacy with a reduced loss of c-peptide levels and insulin usage in the treated cohort compared to placebo treated groups. Phase II studies of patients with new onset T1D treated with a second αCD3 monoclonal antibody, otelixizumab also showed preservation of c-peptide levels and reduced insulin usage in comparison to placebo treated groups. However, side effects of this treatment included reactivation of Epstein-Barr virus (EBV) in 75% of treated patients. Two phase III studies; DEFFEND-1 and DEFFEND-2, were subsequently conducted using lower doses of otelixizumab. In the DEFFEND-1 study, there was no significant difference in c-peptide levels observed between drug treated and placebo treated groups and enrolment in the DEFFEND-2 study was suspended (Ambery et al., 2014; Aronson et al., 2014). In both phase III studies, the reduced dosage had a significant decrease in serious adverse events compared to the phase II studies but correlated with a reduced efficacy. Therefore, further studies are required to improve the balance between efficacy and adverse events with this treatment. In addition to the αCD3 mAbs trials, Rituximab, an αCD20 mAb and Abatacept, a CTLA-4-Ig fusion protein, have been trialled as treatments for T1D. Rituximab functions by depletion of $CD20^+$ B cells and in a phase II study could partly improve insulin secretion over 1 year in comparison to the placebo treated cohort (Pescovitz et al., 2009). Abatacept modulates T cell co-stimulation by binding to CD80 and CD86 co-stimulation molecules and preventing “signal two” – a requirement for T cell activation. Continuous treatment with Abatacept for two years of subjects newly diagnosed with T1D slowed the decline of β cell function by an average of 9.6 months compared to the placebo treated group (Orban et al., 2011). The significant decrease in c-peptide decline was associated with the expansion of naïve $CD4^+$ T cells and the subsequent contraction of the $CD4^+$ central memory population (Orban et al., 2014). However, these changes in $CD4^+$ T cell populations were reversed upon Abatacept administration. Therefore, it is suggested that the continuous use of Abatacept can modify the $CD4^+$ T cell compartment of T1D patients with the effect of slowing down disease progression.

The aim of Ag specific immunotherapy is to promote an immunomodulatory response to certain Ags that are targets in autoimmunity. The administration of whole islet Ag or peptides have shown efficacy in the treatment of murine models of diabetes. The proposed mechanism of peptide immunotherapy is the promotion of IL-10 secreting $CD4^+$ T cells that are activated by immature DCs that present the administered peptide (Tarzi et al., 2006). The induced IL-10 secreting $CD4^+$ T cells can then suppress islet Ag specific responses. In 2009, a first in man phase I clinical trial assessed the safety of proinsulin peptide immunotherapy in the treatment of patients with T1D (Thrower et al., 2009). Most interestingly, this study identified an induction of proinsulin specific IL-10 secreting $CD4^+$ T cells in individuals treated with peptide, although these cells were short-lived. Peptide immunotherapy trials have also been performed using DiaPep277, an Ag derived from hsp60sp. Pathogenic T cell responses to this peptide have been found in both the NOD mouse and in patients newly diagnosed with T1D (Abulafia-Lapid et al., 1999). Administration of this peptide has found to induce Ag specific IL-10 secreting $CD4^+$ T cells and enhance the function of T_{REG} cells via signalling through toll like receptor-2 (Huurman et al., 2008; Zanin-Zhorov et al., 2006). Furthermore, a recently completed phase III clinical trial reported that administration of this peptide could preserve β cell function and improve glycemic control (Raz et al., 2014).

IL-2 is crucial for T_{REG} cell function and development. The high levels of CD25 on the surface of T_{REG} cells, endow an increased sensitivity to IL-2 compared to other immune cell subsets such as T_{EFF} cells or NK cells. It is therefore hypothesised that low dose therapy of IL-2 will selectively target T_{REG} cells for activation and improved function. In fact, low dose IL-2 therapy has been successful in the treatment of GVHD and autoimmune vasculitis (Koreth et al., 2011; Saadoun et al., 2011). In both settings, low dose IL-2 therapy selectively increased T_{REG} cell number without any alteration in the T_{EFF} cell compartment. Importantly clinical improvements were seen in treated patients in both trials and no adverse events of treatments were reported. Pre-clinical efficacy of this treatment has previously

been shown in the NOD mouse model and subsequently low dose IL-2 therapy is currently being investigated as a potential treatment for T1D. Specifically, a reversal of diabetes was seen in 60% of NOD mice treated with low dose IL-2 therapy at the time of disease establishment (Grinberg-Bleyer et al., 2010). This reversal in disease was concurrent with an increase in T_{REG} cell frequency within the islets. However, no disease reversal was identified in NOD mice that were deficient in T_{REG} cells thus identifying the specific effect of this therapy on T_{REG} cells (Grinberg-Bleyer et al., 2010). Rapamycin (RAPA), an immunosuppressive drug, blocks the activation of the mammalian target of Rapamycin complex 1 (MTOR-1), which preferentially inhibits proliferation of T_{EFF} cells. The use of this drug to treat patients with T1D was found to improve the suppressive capacity of T_{REG} cells (Monti et al., 2008b). The combination therapy of low dose IL-2 and RAPA was found to prevent the spontaneous occurrence of T1D in the NOD mice and the reoccurrence of diabetes in mice that were subject to islet transplantation (Rabinovitch et al., 2002). Based on these successes, a phase I safety clinical trial assessing the use of low dose IL-2 therapy with RAPA has now been completed (Long et al., 2012). However, despite the promising pre-clinical data, this trial observed a transient decrease in β cell function and no overall improvement of function over time. Additionally, a large rise in the frequency of NK cells and eosinophils was observed. Subsequently, the additional use of RAPA in this trial was stopped in the last few patients in the treatment group. The study however, did observe a transient increase in T_{REG} cell frequency and an overall improvement of IL-2R signalling was discovered in $CD25^{+}$ cells a year after treatment. Two further "ultra" low dose IL-2 therapy trials are currently underway to determine the optimal dosage of IL-2 for the treatment of T1D. The adaptive study of IL-2 dose of T_{REG} cells in T1D (DILT1D) study has recently completed and aims to identify the doses of IL-2 that achieve a minimum or maximum T_{REG} cell increase in adults aged 18-50 (ClinicalTrials.gov identifier NCT01827735) (Waldron-Lynch et al., 2014). The dose finding study of IL-2 at ultra-low dose in children with recently diagnosed T1D (DFIL2-child) study is also aiming to find the optimal dose of IL-2 to treat T1D

patients but is selectively enrolling children aged 7-12 years of age (ClinicalTrials.gov identifier NCT01862120).

The immunotherapies described have all aimed to strengthen the immune regulation of patients with T1D via a targeted induction of immunosuppressive T_{REG} cells. Although efficacy has been seen, these therapies can elicit off target effects and serious adverse events. An alternative route to strengthening the immune regulation of patients with T1D and improving T_{REG} cell frequency and function is in the form of adoptive T_{REG} cell therapy, which will be the focus of this thesis.

1.3. Adoptive Regulatory T Cell Therapy

1.3.1. Principles of Adoptive Regulatory T cell Therapy

The potential of so-called "suppressor T cells" as a therapeutic tool was first suggested nearly 40 years ago (Gershon, 1975). Following on from this, seminal work in 1995 by *Sakaguchi et al* reported that nude mice reconstituted with $CD25^+$ depleted T cells developed widespread autoimmunity, which was reversed upon re-introduction of the $CD25^+$ T cell compartment (Sakaguchi et al., 1995). Despite nearly four decades of study, therapies aimed at harnessing the suppressive function of T_{REG} cells have only been implemented in the past 10-15 years. In addition to the IL-2 trials discussed above, a number of trials have aimed to modulate the frequency or suppressive effects of T_{REG} cells *in vivo*. The Phase I safety clinical trial that tested the super agonist αCD28 antibody, TGN412, had particularly dramatic consequences. In pre-clinical testing, this antibody was shown to preferentially activate T_{REG} cells and could suppress autoimmunity in a number of mouse disease models (Beyersdorf et al., 2006). However, all participants treated with this drug developed multi-organ failure due to non-targeted activation of all T cells subsets and not just the intended T_{REG} cells (Suntharalingam et al., 2006). Other therapies, such as low dose IL-2 therapy, which is aimed at strengthening and increasing the T_{REG} cell compartment of patients with T1D, have also had off target effects (Long et al., 2012). These data highlight the risks in the

augmentation of a specific immune cell subset *in vivo,* particularly the T_{REG} cell subset, which so far has no unique identifying marker.

An alternative approach to harnessing the suppressive function of T_{REG} cells and increasing their frequency *in vivo* is the use of adoptive T_{REG} cell therapy. This therapy, put simply is the isolation, activation, expansion and/or modification of T_{REG} cells *in vitro* and the re-infusion of the cells back into patients (Figure 1-4). The use of adoptive T_{REG} cell therapy has stemmed from adoptive T cell therapy, which has been used successfully to increase the immune response to cancers and infectious diseases. Although these therapies have only recently been introduced into clinical trials, the principle of adoptive cell therapy is relatively old in terms of immunological based therapies. Studies in the 1950's identified that the adoptive transfer of cells from the tumour draining lymph node of one mouse could mediate immunity to the tumours of a second mouse (Billingham et al., 1954). From these studies, the phrase "adoptive immunity" was coined and the benefits of adoptive T cell therapy was realised. These therapies were further enhanced by the discovery of IL-2 which permitted the *ex vivo* expansion of human T cells (Lotze et al., 1980). This discovery has allowed the field of adoptive T cell therapy to progress and to date, the treatment of melanomas with *ex vivo* expanded tumour infiltrating lymphocytes have yielded promising results (Rosenberg et al., 2008). Adoptive T cell therapy is also being investigated in the treatment of patients with human immunodeficiency virus (HIV) (Riddell and Greenberg, 1995).

With the successes of adoptive T cell therapy at enhancing anti-tumour and anti-pathogen responses, investigators are now researching the possibility of using adoptive T_{REG} cell therapy to promote tolerance in autoimmunity and transplantation.

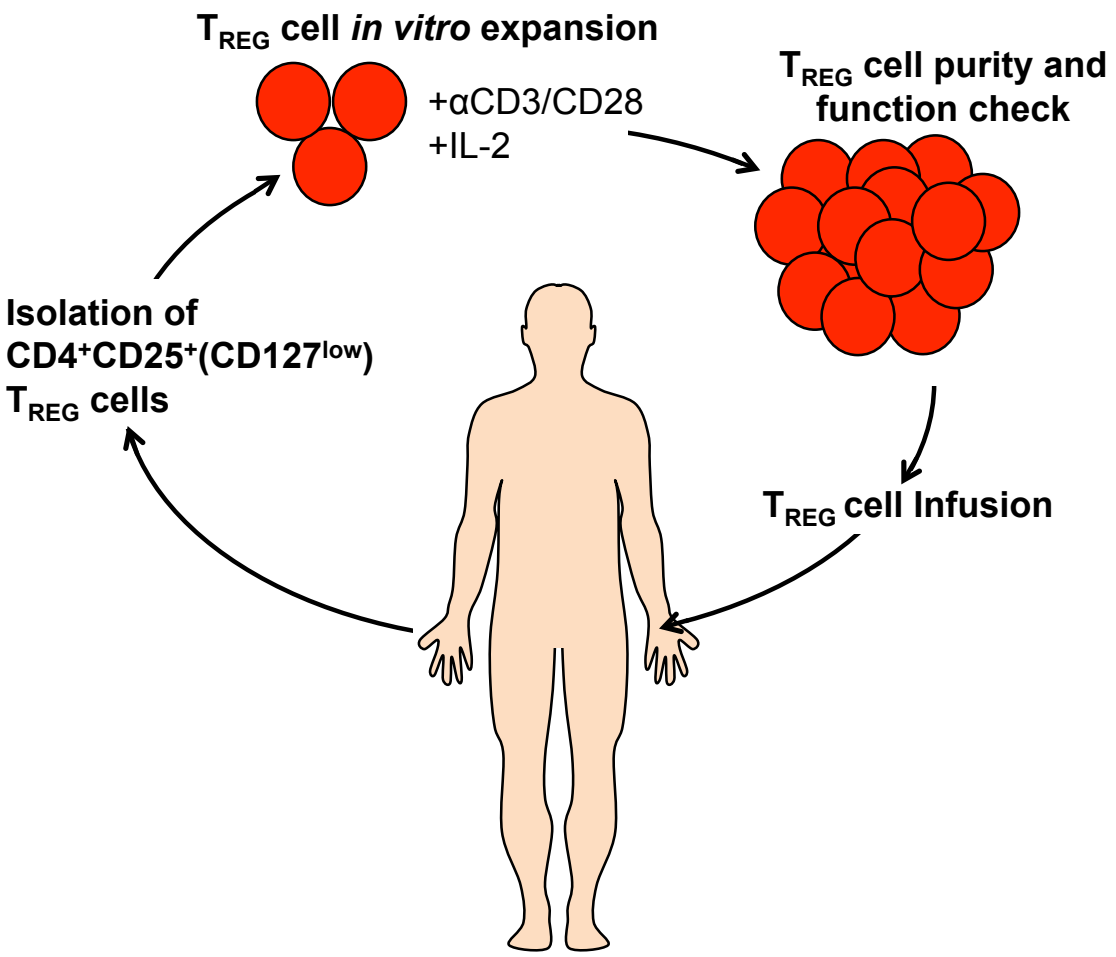

Figure 1-4 Principle of Adoptive T_{REG} cell Therapy. T_{REG} cells can be isolated from the peripheral blood of patients by magnetic bead sorting using the markers $CD4^+$ and $CD25^+$ or by Fluorescent activated cell sorting (FACS) of $CD4^+CD25^+CD127^-$ cells. T_{REG} cells can be expanded using αCD3/CD28 antibodies and a high dose of IL-2. After the *in vitro* expansion period, cells can be tested for purity (including sterility of final product) and their ability to suppress *in vitro*. The final T_{REG} cell product can then be infused into the patient or cryopreserved for infusion later.

1.3.2. Efficacy of Adoptive T_{REG} cell therapy from Pre-Clinical Models

A number of animal models have so far demonstrated efficacy using adoptive T_{REG} cell therapy in the promotion of immune tolerance in the context of GVHD, solid organ transplantation and autoimmunity. However, this therapy is limited by the low frequency of T_{REG} cells, ~5-10% total $CD4^+$ T cells that are found in the periphery

of both mice and humans. Therefore, protocols have been designed for the *ex vivo* expansion of these cells to ensure sufficient cell numbers. These protocols typically involve a polyclonal TCR stimulus such as αCD3/CD28 mAb and high doses of IL-2 to promote expansion and survival of the T_{REG} cells (Tang et al., 2004).

A model of GVHD was the first in mouse disease to be successfully treated with the adoptive transfer of T_{REG} cells (Taylor et al., 2002). GVHD is a lethal disease that occurs when alloreactive T cells from a donor bone marrow graft reacts against the host's MHCI and MHCII molecules. Models of GVHD use the transfer of $CD4^+$ T cells from an MHC mismatched mouse to a second immunodeficient mouse to induce disease. The study by *Taylor et al* identified that the co-transfer of an equal number of *ex vivo* expanded and activated $CD4^+$ T_{REG} cells prevented GVHD mediated by the adoptive transfer of $CD4^+$ T_{EFF} cells. This study identified that *ex vivo* activated T_{REG} were potent suppressors of immune cell mediated disease. The efficacy of the adoptive transfer of T_{REG} cells has also been shown in solid organ transplantation models; this was first demonstrated in a cardiac allograft rat model whereby the adoptive transfer of $CD4^+$ T cells from rats with long term survival of an allografts, promoted tolerance to rats receiving mis matched heart transplants (Hall et al., 1990). More recently, adoptive T_{REG} cell therapy was shown to promote the tolerance of a porcine islet transplant in a humanised mouse model (Yi et al., 2012).

It is widely recognised that T_{REG} cells require activation through their TCR before eliciting suppressive function, however the subsequent suppressive mechanisms can act in a non-Ag specific manner (Thornton and Shevach, 2000). Although the T_{REG} cells are activated *in vitro* prior to infusion into mouse models/patients, it is hypothesised that these cells will require further TCR activation *in vivo* to elicit full suppressive function. Thus, it is widely suggested that adoptive T_{REG} cell therapy using Ag specific T_{REG} cells would be more efficacious than polyclonal T_{REG} cells, and indeed this has been investigated in a number of mouse models. A study by *Tang et al* in 2004 demonstrated the superior potency of Ag specific T_{REG} cells by

using the BDC2.5 TCR Transgenic mouse (Tang et al., 2004). This mouse is transgenic for the TCR genes of the BDC2.5 $CD4^+$ T cell clone, which was isolated from the NOD mouse and has been shown to be specific for a peptide of islet Ag chromogranin A (Stadinski et al., 2010). Isolated and expanded BDC2.5 T_{REG} cells were found to suppress the transfer of diabetes by diabetogenic T cells in the immunodeficient NOD.$RAG^{-/-}$mice (Tang et al., 2004). Moreover, in comparison to polyclonal T_{REG} cells derived from a NOD mouse, a much lower number of Ag specific T_{REG} cells were required to mediate this suppression. Furthermore, the transfer of Ag specific T_{REG} cells could prevent the rejection of transplanted syngeneic islet cells in a NOD mouse. In the same model, the transfer of expanded polyclonal T_{REG} cells could not promote tolerance to the transplant. The study by *Tang et al* expanded BDC2.5 T_{REG} cells using αCD3/CD28 stimulation and high dose IL-2 but it has been identified that Ag specific T_{REG} cell can also be expanded using peptide pulsed DCs in combination with high dose IL-2 (Yamazaki et al., 2003). Two subsequent studies identified that BDC2.5 T_{REG} cells expanded with cognate peptide pulsed DCs were capable of suppressing diabetes onset in a diabetes transfer model (Tarbell et al., 2007; Tarbell et al., 2004). The first study also identified that the Ag specific T_{REG} cell were >100 fold more potent at preventing diabetes development than polyclonal T_{REG} cells (Tarbell et al., 2004). Furthermore, the Ag specific T_{REG} cells were able to prevent diabetes development in pre-diabetic mice and delay development in recent onset diabetic NOD mice (Tarbell et al., 2007). Interestingly, the second study identified that the successfully treated NOD mice retained diabetogenic T cells in the pancreatic draining lymph node and pancreas but had an increased frequency of T_{REG} cells that were host derived (Tarbell et al., 2007), suggesting that the adoptively transferred T_{REG} cells were able to promote a regulatory environment at the site of inflammation leading to long term protection.

1.4. Moving Adoptive T_{REG} cell therapy into Man

1.4.1. T_{REG} cell selection and Expansion

One of the main drawbacks to adoptive T_{REG} cell therapy in humans is the lack of a definitive marker for this cell subset. As previously mentioned, these cells are typically characterised as $CD4^+CD25^+FoxP3^+$. However, FoxP3 is an intra-nuclear protein and staining for this marker requires the fixation and permeabilisation of cells. To remove the requirement of staining for FoxP3, the expression of IL-7 receptor α chain, CD127, has been found to inversely correlate with FoxP3 expression (Liu et al., 2006). Therefore, the isolation of $CD4^+CD25^+CD127^{low}$ cells typically yields a greater than >95% pure T_{REG} cell population. However, recently activated T_{EFF} cells can up-regulate CD25 and FoxP3 expression and there is a possibility of contaminating T_{EFF} cells within the isolated T_{REG} cell population. Due to the polyclonal expansion protocols of human T_{REG} cells, contaminating T_{EFF} cells would expand to a greater extent that T_{REG} cells, therefore increasing their frequency in the whole population. Thus, it is vital that the isolation of T_{REG} cells minimised T_{EFF} cell contamination. CD45RA is another marker that can be used to further delineate human T_{REG} cells. Ag stimulated T cells lose expression of CD45RA in favour of CD45RO expression and therefore CD45RA expression can denote naïve T cells. It was further identified by *Miyara et al* that three subpopulations of T_{REG} cells could be delineated by CD45RA and FoxP3 expression (Miyara et al., 2009). T_{REG} cells that are $CD45RA^+FoxP3^{low}$ are found to be "resting T_{REG} cells" and $CD45RA^-FOXP3^{high}$ "effector T_{REG} cells," both these subsets have shown suppressive function *in vitro.* However, cells that are $CD45RA^-FoxP3^{low}$ have been shown to secrete pro inflammatory cytokines such as IL-17, although this has recently been found to not compromise T_{REG} cell suppressive function (Afzali et al., 2013). Additionally, "resting T_{REG}" cells have been shown to have greater suppressive capacity that total $CD4^+CD25^{hi}$ T_{REG} cells. These cells have also been found to remain demethylated at the T_{REG} specific demethylation region (TSDR) within the *FOXP3* gene after *ex vivo* expansion (Hoffmann et al., 2009). Demethylation within this region has been shown to

correlate with *FOXP3 stability* and specific demethylation within this region denotes a stable T_{REG} cell in comparison with recently activated T_{EFF} cells (Baron et al., 2007). However, a caveat with isolation of T_{REG} cells based on CD45RA expression is that the expression of this marker decreases with age (Seddiki et al., 2006).

Another potential problem with adoptive T_{REG} cell therapy in humans is the isolation and expansion of sufficient quantities of cells for effective therapy. A study by *Putnam et al* isolated $CD4^+CD25^+CD127^{low}$ T_{REG} cells from the peripheral blood of patients with T1D using fluorescent activated cell sorting (FACS) and expanded the cells *in vitro* with αCD3/CD28 coated microbeads and high dose IL-2 for two weeks (Putnam et al., 2009). This protocol, on average, yielded a 1500 fold expansion of T_{REG} cells and the cells retained high levels of FoxP3 expression and suppressive capabilities in *in vitro* suppression assays. Moreover, comparison of the expansion potential of $CD45RA^+$ and $CD45RA^-$ T_{REG} cells revealed that the $CD45RA^+$ T_{REG} cells expanded at a greater rate. The benefit of FACS sorting over other methods of cell isolation, such as magnetic beads, is a higher purity and yield of T_{REG} cells (typically 95% for FACS and 60% for magnetic beads) (Peters et al., 2008; Putnam et al., 2009). Additionally, the protocol outlined in the study by *Putnam et al* can be modified to meet good manufacturing practice (GMP), which is vital to therapies using cell products. The addition of RAPA to expanding T_{REG} cell cultures has also been shown to improve the purity of expanded T_{REG} cells by inhibiting the expansion of T_{EFF} cells (Battaglia et al., 2006). However, the addition of RAPA to cultures has been shown to reduce the expansion of T_{REG} cells by >10-fold (Golovina et al., 2008). An alternative source of T_{REG} cells is from umbilical cord blood (UCB) as opposed to peripheral blood. UCB cells are immunologically naïve as the blood cells derive in a protected environment, thus UCB cells lack memory or recently activated $CD25^+$ T_{EFF} cells that may contaminate the $CD25^+$ T_{REG} cell population. $CD4^+CD25^+$ T_{REG} cells isolated from UCB were found to expand ~200-300 fold, be potent suppressor cells and have limited capacity for producing cytokines including IL-2, IFN-γ and IL-10. Although these cells provide an attractive

therapeutic tool, this therapy would largely rely on the use of allogeneic T_{REG} cell transfer, which could open up the possibility of the rejection of transferred cells.

1.4.2. Moving T_{REG} Cell Therapy Into The Clinic

GVHD became the first human disease to be treated by the adoptive transfer of T_{REG} cells. The first study treated two patients, one with chronic GVHD that had persisted two-years post bone marrow transplant and the second had acute GVHD at one-month post transplant (Trzonkowski et al., 2009). The patient with chronic GVHD was treated successfully with a single infusion of *ex vivo* autologous expanded $CD4^+CD25^+CD127^-$ T_{REG} cells that led to complete withdrawal of immunosuppressive drugs. The patient with acute GVHD however, was treated with multiple infusions of expanded donor T_{REG} cells that were unable to prevent the progression of disease. A second phase I/II trial administered expanded $CD4^+CD25^{hi}$ T_{REG} cells from a third party UCB graft to patients receiving UCB stem cell transplants (Brunstein et al., 2011). This study demonstrated the safety and tolerability of the UCB derived T_{REG} cells and although the severity of acute GVHD decreased the overall incidence was not. Currently there is an on-going multicentre phase I/II study investigating the safety of administering *ex vivo* expanded T_{REG} cells to patients receiving kidney transplants (ClinicalTrials.gov identifier NCT02129881).

A phase I safety clinical trial assessing the use of polyclonal T_{REG} cells to treat newly diagnosed (within >3 and <24 month) patients with T1D is ongoing (ClinicalTrials.gov identifier NCT01210664). This study is implementing the polyclonal expansion of FACS isolated $CD4^+CD25^+CD127^{low/-}$ T_{REG} cells with αCD3/CD28 coated beads and IL-2 and will administer T_{REG} cells at a single time point and include a dose escalation study. The primary end-point of this study is the safety and tolerability of T_{REG} cell infusion in T1D although C-peptide response, the use of insulin and immunological markers will be assessed as secondary outcomes. Although this study is not due for completion until December 2016, it should provide valuable data on the feasibility of T_{REG} cell transfer in the treatment

of T1D. A second study that administered FACS sorted $CD4^+CD25^+CD127^-$ T_{REG} cells to children within two months of T1D diagnosis completed in 2012 (Marek-Trzonkowska et al., 2012). This study treated ten children with a single infusion of T_{REG} cells at either $10x10^6$ or $20x10^6$ T_{REG} cells/kg weight. Firstly, the study identified the safety of the transfer of T_{REG} cells and that no significant difference between the two doses of T_{REG} cells was observed. Of the ten children treated with T_{REG} cells, two children remained insulin independent at 6 months, compared to no children in the non-treated cohort. In a follow up study the investigators administered previously treated children with a second dose of T_{REG} cells (Marek-Trzonkowska et al., 2014). The two children who were initially insulin independent after the first dose of T_{REG} cells remained insulin independent after the second dose. In total, 66% of the treated children were in disease remission post T_{REG} treatment, including five treated with two T_{REG} cell infusions, two with a single infusion of $20x10^6$/kg body weight and one with an infusion of $10x10^6$/kg body weight. This was in comparison to 20% of children in the non-T_{REG} cell treated cohort.

1.4.3. Therapy With Ag Specific T_{REG} Cells in Man

The results from the small number of trials assessing adoptive T_{REG} cell therapy using *ex vivo* expanded polyclonal T_{REG} cells have been promising. However, the data is limited and the results from the two on-going trials assessing these cells in T1D and solid organ transplantation should expand the knowledge base on this therapy. It has been shown in animal models that Ag specific T_{REG} cells are more efficacious at suppressing disease than polyclonal T_{REG} cells. The studies by *Marek-Trzonkowska et al* also identified that the therapeutic benefit of T_{REG} cells waned over time and that multiple doses may be required (Marek-Trzonkowska et al., 2014). Evidence from animal studies indicates that treatment with Ag specific T_{REG} cells would require much lower numbers of cells, compared to polyclonal T_{REG} cells, as these cells can be >100 fold more potent. A number of methods that can be used to expand Ag specific populations of T_{REG} cells have been identified (Figure 1-5). As discussed, DCs pulsed with islet Ags have been used to expand

Ag specific T_{REG} cells isolated from mice (Tarbell et al., 2007). However, the frequency of Ag specific T_{REG} cells in peripheral blood is reported to be only 1:20,000-1:200,000 cells making it harder to achieve a suitable number of T_{REG} cells for therapy (Di Lorenzo et al., 2007).

Several methods to generate Ag specific T_{REG} cells by stimulation of naïve $CD4^+CD25^-$ T cells in the presence of cytokines or growth factors have been described. For example, these so called "adaptive T_{REG} cells" can be generated *in vitro* from naïve $CD4^+CD25^-$ T cells by incubation with peptide pulsed DCs and IL-2 (Long et al., 2009; Walker et al., 2005). Adaptive T_{REG} cells can also be generated by exposure to Ag in the presence of TGF-β and RAPA (Chen et al., 2003). However, there are major questions over the stability of adaptive T_{REG} cells with the possibility that these cells can revert to an T_{EFF} cell phenotype under inflammatory conditions (Roncarolo and Battaglia, 2007). Therefore, the use of Ag specific adaptive T_{REG} cells as a treatment to autoimmune disease would pose a high risk as the reversion of these cells at inflammatory sites could worsen disease state. To circumvent issues over low frequency and stability of Ag specific T_{REG} cells, it has been suggested that designer T_{REG} cells can be generated using viral vectors (Brusko et al., 2010; Plesa et al., 2012). This process involves the transfer of TCR genes of any chosen Ag specificity to polyclonal T_{REG} cells with the aim of generating a large population of Ag specific T_{REG} cells. The transfer of TCR genes could also be supplemented with the transfer of genes that can stabilise T_{REG} cell function.

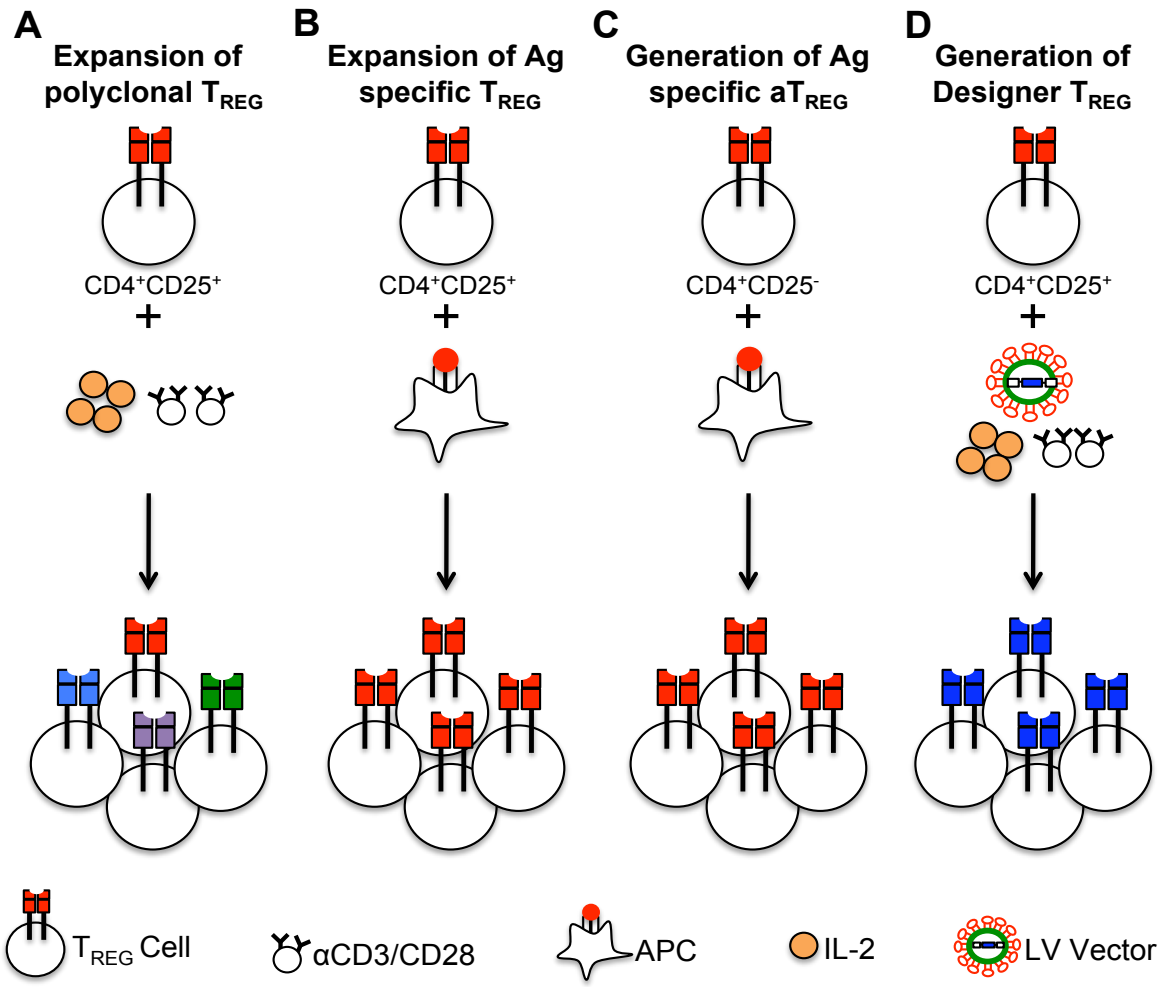

Figure 1-5 Methods to Expand Ag Specific T_{REG} cells. (A) Polyclonal T_{REG} cells can be expanded from isolated $CD4^+CD25^+$ cells using αCD3/CD28 stimulation with high dose IL-2. (B) Peptide pulsed APCs can be used to expand Ag specific T_{REG} cells from $CD4^+CD25^+$ T_{REG} cells and (C) generate *de novo* Ag specific adaptive T_{REG} cells (aT_{REG}) from $CD4^+CD25^-$ naïve T cells. (D) The Ag specificity of activated polyclonal T_{REG} cells can be re-directed using viral vectors to deliver TCR genes. Adapted from *Brusko et al*, Immunol Rev. 2008 (Brusko et al., 2008)

1.5. Gene Therapy Using Viral Vectors

1.5.1. History and Principles of Viral Based Gene Transfer

T-cell engineering is a form of gene therapy in which genetic material is transferred to a target T cell to enhance its therapeutic application. A number of vehicles, referred to as vectors, can be exploited for the transfer of genetic information. A common method involves the use of viruses. Some viruses possess an exquisite

biological mechanism for cell entry and integration into the host DNA, which can be harnessed for the delivery of therapeutic genes. In particular, RNA viruses such as retroviruses, are unique in their ability to reverse transcribe their RNA genome into double stranded DNA (dsDNA) and stably integrate into the genome of the host cell. For T cell engineering, this includes the delivery of specific TCR genes into a T cell in order to re-direct the Ag specificity of a bulk population of cells. This method of T cell engineering has been used to generate large numbers of Ag specific T cells for the treatment of cancers and infectious diseases (Cooper et al., 2000; Morgan et al., 2006; Robbins et al., 2011). Recently, this concept has been applied to the generation of Ag specific T_{REG} cells, which can circumvent the problems associated with the isolation and expansion of rare populations of T_{REG} cells (Brusko et al., 2010).

The earliest viral vectors for gene therapy generated were γ-retroviral vectors based on the Moloney Murine Leukaemia Virus (MoMLV) and research on these vectors peaked in the 1980's (Friedmann, 1992). The main principle of retrovirus engineering for gene therapy is to separate viral components required for DNA replication from those that cause disease. Therefore, genes that are required for viral entry, replication, assembly and packaging and genes that encode immunogenic Ags were deleted from the MoMLV viral genome. The remaining viral genome was then used as a backbone to clone in any gene of interest, referred to as a transgene (Mann et al., 1983) (Figure 1-6). The resulting construct, known as the transfer plasmid, retains *cis* acting genomic sequences known as long terminal repeats (LTRs), which encode all the genes crucial for the transcription, integration and expression of the transgene. Importantly, the transfer vector does not encode any genetic information for viral replication or the packaging of virions. Rather, the helper genes required for the production of virions are provided in *trans* by two separate DNA plasmids (Markowitz et al., 1988). The envelope plasmid encodes the genes for the viral envelope and subsequently provides the tropism of the virus. The second helper plasmid, referred to as the packaging plasmid, encodes the genes required for the virus structure and viral replication (Figure 1-6). Importantly,

the transfer plasmid is the only plasmid to retain the Ψ sequence, which directs viral RNA encapsidation (Cooray et al., 2012). Therefore, only the transgene is packaged into the virus capsid and released into the host cell cytoplasm upon virus infection. These replication incompetent retroviruses can be generated by transient transfection of the transfer plasmid and two helper plasmids into a packaging cell line. The recombinant virus is then released into the packaging cell supernatant and can be used for the transduction of a plethora of host cells. Transduction refers to the infection of host cells with replication incompetent virus as opposed to a productive virus infection.

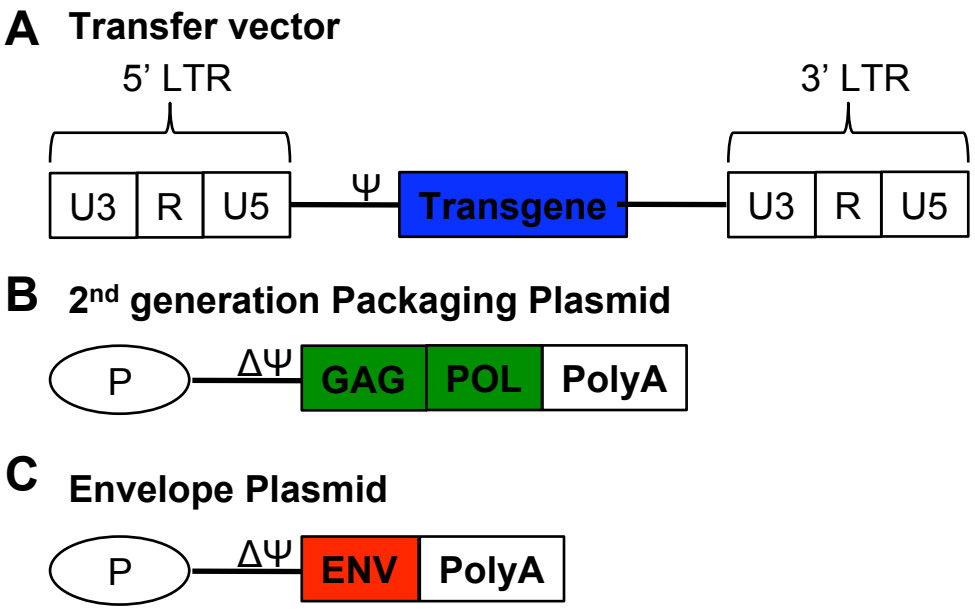

Figure 1-6 First Generation γ-Retroviral Vectors Derived from MoMLV. (A) The transgene vector is cloned into the transfer plasmid that contains the viral derived long terminal repeats (LTR) consisting of a U3, R and U5 domain. The 5' U3 region contains promoter and enhancer sequences that are crucial to reverse transcription and the 3' U5 region allows for transcriptional termination. The R region is vital to the strand transfer that occurs during reverse transcription. The transfer plasmid also contains the Ψ sequence, which is essential for viral RNA encapsidation (Cooray et al., 2012). (B) The packaging plasmid encodes the *gag* gene, which provides the structure of the virus and the *pol* gene, which allows for viral DNA replication. Both genes are transcribed by an internal promoter (P) (C) The Envelope Plasmid encode the virus envelope, *env* gene and determines the tropism of the virus The Ψ sequence is removed (ΔΨ) in both the envelope and packaging plasmid to ensure only the transfer plasmid DNA is encapsulated in the resulting retrovirus.

For productive transgene expression, the proteins in the envelope of the virus must first interact with its cognate receptor on the host cells to gain entry into the cell (Figure 1-7).

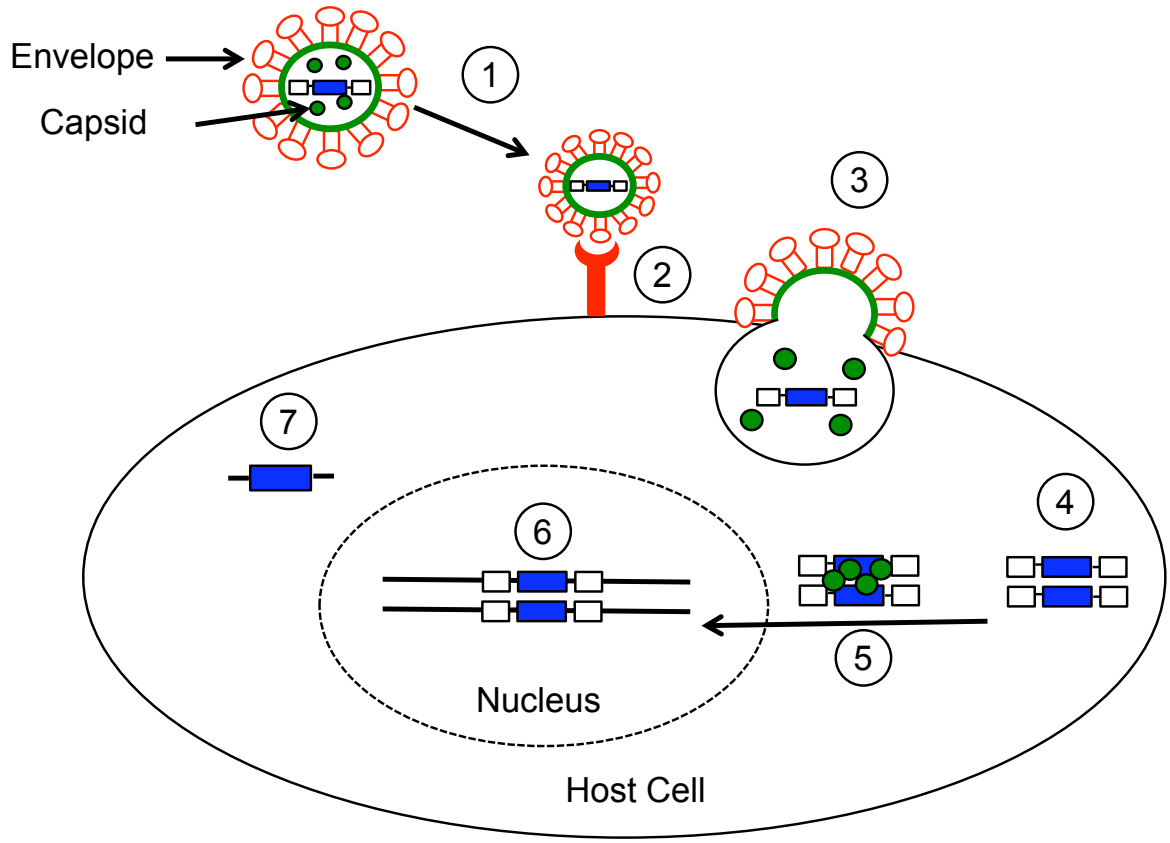

Figure 1-7 Schematic Representation of Recombinant Retroviral Mediated Gene Transfer. (1) The replication incompetent virus is constructed from the genes of three separate plasmids, envelope, packaging and transfer. The viral capsid contains the viral RNA encoding the transgene and the reverse transcriptase and integrase enzymes encoded by *pol.* (2) The proteins in the viral envelope interact with their cognate receptors on the host cell surface. (3) The virus and host cell membranes fuse and the viral capsid containing the vector RNA and viral enzymes are released into the host cell cytoplasm. (4) The vector RNA transgene is reverse transcribed to generate dsDNA, and is now known as the provirus (5) The provirus associated with the viral enzymes forming a pre-integration complex (PIC) and translocates to the nucleus during cell division when the nuclear membrane is permeable. (6) The provirus is integrated into the host DNA. (7) The LTRs act as a promoter and the vector DNA is transcribed by the host machinery and expressed in the host cell.

As the genes for the envelope are provided in *trans*, the envelope plasmid can encode the *ENV* gene of any retrovirus, which allows the virus to be pseudotyped. For example, viruses are commonly pseudotyped with the envelope glycoprotein from the pan tropic Vesicular Stomatitis Virus (VSV), which interacts with the low density lipoprotein receptor - a receptor ubiquitously present in the cell membrane (Agnello et al., 1999). Thus the pseudotyping of recombinant retrovirus with an envelope such as VSV-G, provides the virus with a selective advantage as it increases the pool of host cells that it can infect.

The first clinical trial to use cells modified by viral vectors was carried out in 1990. In the first trial, *Rosenberg et al* treated five advanced melanoma patients with the adoptive transfer of tumour infiltrating lymphocytes that had been modified using retroviral vectors (Rosenberg et al., 1990). The cells were modified to express a neomycin resistance gene, which permitted the tracking of these cells *in vivo*. The main success of this study was the absence of serious adverse advents due to the gene modified T cells. All patients were tested for the presence of replicating virus post T cell transfer to ensure the virus remained replication incompetent, all tests were negative. Interestingly the gene-modified cells could be detected in the circulation and tumour biopsies over 50 days after T cell therapy.

In more recent years, $CD34^+$ stem cells from patients with adenosine deaminase (ADA)-SCID have been transduced *ex vivo* to express the ADA gene. So far, two thirds of the ADA-SICD patients who have been treated with ADA expressing $CD34^+$ cells have shown clinical benefit with no adverse events (Aiuti et al., 2009). Another variant of SCID, known as SCID-XI, is caused by a loss of function mutation in the gene encoding the IL-2R common γ chain, *IL2RG*. Clinical trials completed in London and Paris used retroviral vectors to express a functioning *IL2RG* gene in $CD34^+$ stem cells and transfuse these cells back into patients. The treatment was successful in 18 out of 20 patients; however, five of the patients developed T cell leukaemia after treatment (Howe et al., 2008). Subsequent research found that the retroviral vectors favoured integration within active genes

such as proto-oncogenes. It was also identified that the enhancer regions within the LTRs of the virus could permanently activate the transcription of the active gene leading to the ensuing T cell leukaemia.

1.5.2. Benefits of Lentivirus Derived Viral Vectors for Gene Therapy

The insertional mutagenesis observed in the SCID-XI trials, which used MoMLV retroviral based vectors, highlighted the safety concerns of such vectors. Investigators have demonstrated that the safety of these vectors can be improved by deletion of the enhancer regions, U3, in the 3' LTR (Yu et al., 1986). During reverse transcription, this deletion is transferred to the 5' LTR, which results in transcriptional inactivation. These vectors, known as self-inactivating (SIN) vectors, require the use of an internal promoter for transgene expression. Lentiviral (LV) vectors such as those derived from HIV-1 have also been employed for use in gene therapy. These vectors are similar to γ-retroviral derived vectors in that they can stably integrate within the host genome to provide continuous transgene expression. However, LV derived vectors have the additional benefit of being able to infect quiescent cells (Naldini et al., 1996). Furthermore, unlike γ retroviral vectors, LV derived vectors have a tendency to integrate within active genes and not at transcriptional start sites, therefore reducing the chances of insertional mutagenesis (Schroder et al., 2002; Wu et al., 2003). An additional advantage that LV vectors have to γ-retroviral vectors is their larger packaging capacity of up to 10kb, although increasing transgene size can have a negative effect on viral titre thereby reducing transduction efficiency (Sinn et al., 2005).

LVs represent more complex viruses than γ-retroviruses and encode additional regulatory genes, *tat* and *rev* and accessory genes *vpr*, *vif*, *vpu* and *nef* (Amado and Chen, 1999). *Rev* interacts with the rev responsive element (RRE) to enable the nuclear export of viral mRNA into the cytoplasm, whilst *tat* activates the promoter of the LTR to enabling an increased efficiency in provirus production. The first generation SIN LV vectors consisted of a transfer plasmid, a packaging plasmid expressing *gag* and *pol* as well as the six LV associated genes and an

envelope plasmid. The transfer plasmid incorporated additional elements that were not found in γ-retroviral derived transfer vectors. These included the central polypurine tract (cPPT), which facilitated the nuclear translocation of HIV PICs and a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) that can increase viral RNA stability and subsequently transgene expression (Sinn et al., 2005). In a bid to improve the safety of LV vectors, investigators identified that the removal of the four accessory genes (*vpr*, *vif*, *vpu* and *nef*) from the packaging plasmid did not affect the production of LV or transduction by LV of a majority of cell types (Zufferey et al., 1997). These modified vectors, known as second generation packaging plasmids, significantly improved the safety of LV vectors as any potential replication competent LVs generated by recombination events would be devoid of the necessary HIV virulence factors (Figure 1-8).

Further improvements in the safety of these vectors have seen the construction of third generation LV vectors that use two packaging plasmids for LV production. In these packaging plasmids *rev* is separated from *gag* and *pol* and provided in *trans* on a separate plasmid. Furthermore, *tat* is removed from the packaging plasmids as third generation LV transfer vectors possess a chimeric 5'LTR, that encodes a constitutive viral promoter in the place of the 5' U3 enhancer region (Dull et al., 1998) (Figure 1-8). The constitutive promoter provides sufficient transcription rendering the *tat* gene redundant. The third generation LV system therefore has added safety benefits as the use of two packaging plasmids instead of a single one reduces the likelihood of recombination events resulting in replication competent LVs. It is important to note that third generation LV transfer vectors can use either the second or third generation packaging plasmids.

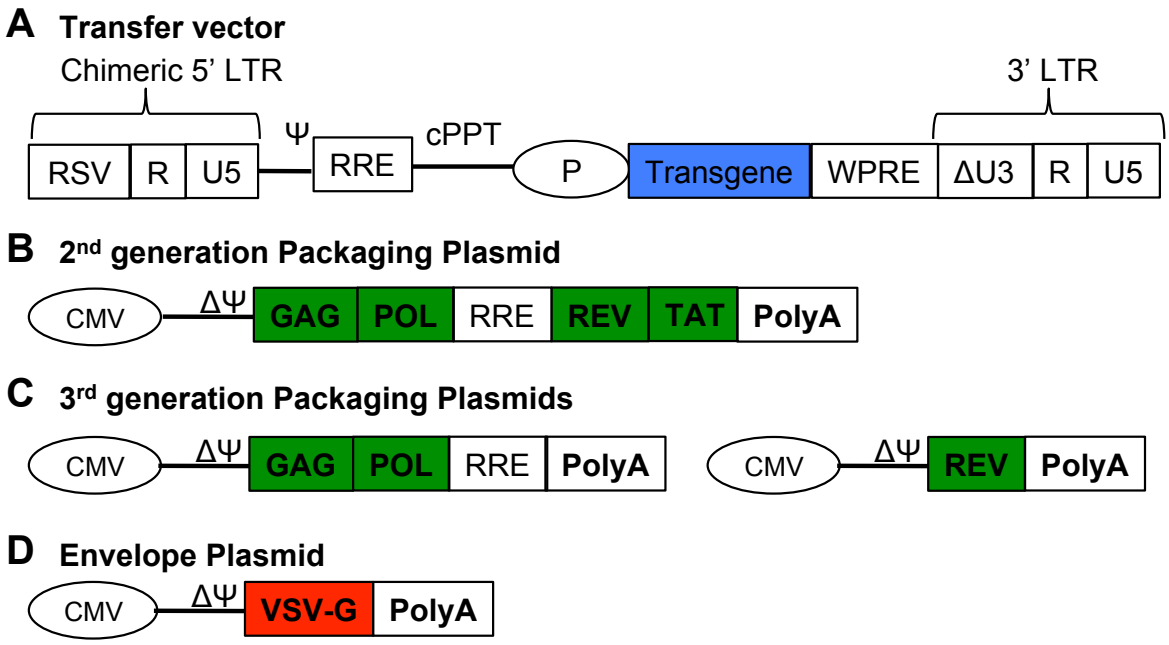

Figure 1-8 Schematic of second & third Generation SIN LV Transfer vectors. (A) The third generation SIN transfer vector consists of a chimeric 5' LTR that replaces the U3 enhancer region with the Rous Sarcoma Virus (RSV) promoter, whereas the U3 region of the 3' LTR is deleted (ΔU3). An internal promoter is used to drive the expression of the transgene upon incorporation into the hosts' chromosome. (B) A second generation packaging plasmid consists of a single plasmid and (C) a third generation packaging plasmid consists of a packaging plasmid and a *rev* expressing plasmid. (D) LV vectors for gene therapy are commonly pseudotyped using the envelope gene of VSV to broaden the virus tropism. P – promoter, RRE – rev responsive element, cPPT – central polypurine tract, WPRE - woodchuck hepatitis virus post-transcriptional regulatory element, CMV – cytomegalovirus promoter.

1.6. T Cell Engineering

The transfer of Ag specific T cell receptor genes to a different T cell of unknown specificity provides an elegant solution to the generation of large numbers of Ag specific T cells. As previously mentioned the Ag specificity of a T cell is conferred by its expression of a TCR, which recognises its specific peptide in complex with MHC molecules. A landmark study by *Dembic et al* was the first to identify that the expression of TCR genes from one T cell could confer the TCRs Ag specificity to a donor T cell (Dembic et al., 1986). Following on from this, a number of groups have

successfully re-directed the Ag specificity of $CD4^+$ and $CD8^+$ T cell populations by the transfer of viral and tumour Ag specific TCRs (Clay et al., 1999; Cooper et al., 2000). In addition to TCR gene transfer, T cells have also been engineered to express artificial Ag receptors. These receptors, known as chimeric Ag receptors (CARs), combine both Ag recognition and immune receptor signalling.

There are three main challenges with TCR gene therapy. Firstly, the introduced TCR must have an optimal avidity for its cognate pMHC to allow productive TCR signalling. This avidity is determined by the overall affinity that the TCR has for cognate pMHC as well as its expression levels on the surface of the introduced cell. Furthermore, the engineered T cell must be long-lived to ensure their therapeutic effect is met and the safety of the cells must be absolute with no off or on target toxicity.

1.6.1. T Cell Receptor Composition

Over 95% of circulating T cells possess a TCR comprised of a TCRα and TCRβ chain. The TCRα and TCRβ chains consist of a TCRα or TCRβ constant (C) domain linked to a vα or vβ domain (Figure 1-9). The V domains undergo V(D)J recombination in the thymus during T cell development and provide the Ag recognition site of a TCR. Within the vα and vβ domains are hypervariable complementary determining regions (CDR) known as CDRs 1-3 and the highest variability is found in CDR3. Although the TCR is a heterodimer, the genes that encode the TCRα and TCRβ chains are continuous, which allows for cloning of each chain into a viral vector. Therefore, the genes that encode a TCR can be transferred to T cells using viral vectors in the same manner described previously for other therapeutic genes.

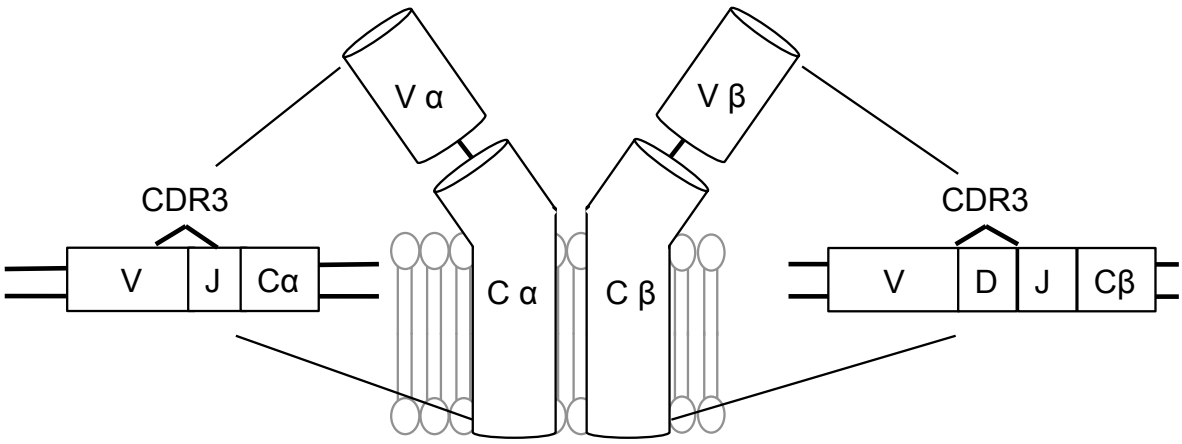

Figure 1-9 Schematic Diagram of TCR structure. A TCR is comprised of a TCRα and TCRβ chain. Both the TCRα and TCRβ chain have conserved constant (C) domains, Cα and Cβ respectively as well as variable domains, the structural arrangement of which provides the Ag recognition site. The Vα chain is comprised of a linked Vα and Jα domain, whereas the Vβ chain has an added D domain. The complimentary determining region 3 (CDR3) provides the most variability within the Ag recognition site.

The composition of viral vectors encoding TCR genes is essential to the success of TCR gene therapy. To ensure that the avidity of the interaction between the introduced TCR and cognate pMHC is not compromised, the expression of the introduced TCR must be maximised. Additionally, equimolar translation of both the TCRα and TCRβ genes is required for optimal expression and pairing of the introduced TCR chains. The original viral vectors for TCR gene therapy either expressed the two TCR chains in separate viral vectors or utilised a single vector with two internal promoters. Additionally, the inclusion of an internal ribosome entry site (IRES) between the two TCR chains generated multicistronic vectors allowing for expression of both the genes under control of the same promoter. However, all of these methods have been linked to problems of poor TCR expression due to inequalities in the translation of the TCR chains (Mizuguchi et al., 2000). To circumvent these problems, multicistronic vectors have been generated that incorporate a self-cleaving 2A peptide derived from the Picornaviridae family of viruses (Szymczak et al., 2004). The 2A peptides contain a consensus motif that acts as a ribosomal skip site thereby preventing the formation of a peptide bond between adjoining protein-coding sequences. Therefore, the 2A peptide sequences

can link several genes within a single open reading frame resulting in equimolar translation of the genes. Furthermore, the use of these sequences has been shown to improve pairing and expression of introduced TCR chains by up to four fold in comparison to the use of an IRES (Wargo et al., 2009).

1.6.2. TCR signalling

The cytoplasmic tails of the TCRα and TCRβ chain are very short and do not possess any signalling domains. Therefore, the TCR forms a complex with a group of molecules collectively known as CD3. The CD3 signalling complex is made up of four proteins, γ, δ, ε and ζ, all of which possess long cytoplasmic tails that between them contain ten immunoreceptor tyrosine-associated based activation motifs (ITAMs). Upon TCR recognition of its specific pMHC complex, the tyrosine kinase, Lck is recruited to the TCR-CD3 complex where it can phosphorylate the ITAMs on the CD3 proteins, enabling the recruitment of the ζ-chain associated protein kinase of 70kDa (Zap70), which is activated by Lck mediated phosphorylation. Activated Zap70 can in turn phosphorylate the tyrosine residues on the linker for activation of T cells (LAT), which subsequently recruits a number of downstream signalling molecules resulting in T cell activation (Figure 1-10) (Brownlie and Zamoyska, 2013).

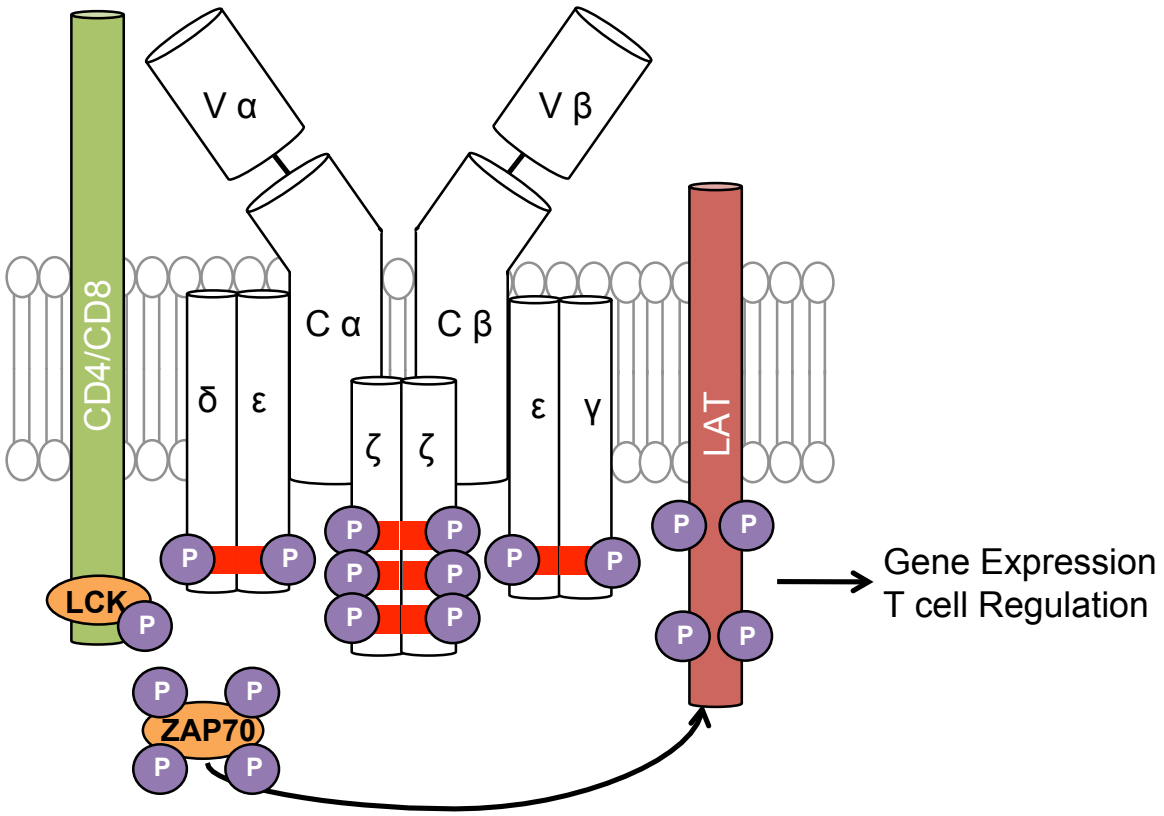

Figure 1-10 Schematic of T Cell Receptor Signalling. The TCR is associated with the δ, ε, γ, and ζ chains of the CD3 signalling complex. Upon TCR engagement by pMHC the T cell co-receptor (CD4 or CD8) recruits Lck to the CD3 signalling complex and phosphorylates the ten ITAMS. Zap70 is recruited to the complex and becomes phoshorylated and activated by Lck. Activated Zap70 phosphorylates key residues on the linker for activation of T cells (LAT) and a number of signalling molecules are recruited leading to downstream gene expression and T cell regulation.

$CD8^+$ T cells and $CD4^+$ T cells are restricted by MHCI and MHCII pMHC complexes respectively and are aided in the recognition of pMHC by the expression of the CD8 and CD4 co-receptors. $CD8^+$ T cells recognise pMHCI complexes that are present on every nucleated cell on the body, whereas MHCII expression is normally limited to professional APCs, such as DCs and other immune cells. The CD8 co-receptor is most commonly expressed as a CD8αβ heterodimer but can also be expressed as a CD8αα homodimer. The CD8αβ co-receptor specifically is important in stabilising the interaction between TCR and pMHC (Wooldridge et al., 2005).

CD4 is a single chain co-receptor and, unlike CD8$\alpha\beta$, is not thought to play a role in stabilising TCR and pMHCII complexes. However, both co-receptors are involved in the recruitment of Lck to the TCR signalling complex (Artyomov et al., 2010). An elegant study by *Holler et al* performed a quantitative analysis on the contribution of CD8 to the overall affinity of a TCR and pMHC interaction (Holler and Kranz, 2003). The authors expressed a panel of TCRs of varying affinities for the same Ag in a TCR$\alpha\beta^-$ hybridoma and tested the sensitivity of the TCRs for peptide in the presence and absence of the CD8 co-receptor. The data from these experiments identified that the highest affinity TCR could be activated by pMHCI in the absence of CD8, whereas the lowest affinity TCR pMHC interaction was dependent on CD8. Furthermore, the highest affinity TCR could be activated with 1000 fold lower concentration of peptide that the TCRs with lower affinity for pMHCI. It was therefore identified that CD8 can augment sensitivity of a low affinity TCR pMHC interaction. Moreover, it was identified that high affinity TCR-pMHCI interactions could lead to productive T cell activation without the requirement of CD8, i.e. were CD8 independent, suggesting that both the affinity between a TCR and pMHC and co-receptor presence on a T cell are important factors to consider in T cell receptor gene therapy.

1.6.3. Chimeric Antigen Receptors

Pioneering studies by *Gross et al* sought to engineer T cells to recognise Ags in a non MHC restricted manner. During these investigations an antibody derived single chain variable fragment (scFv) was fused to the signalling domain of a TCR to generate a chimeric receptor, which was found to signal in response to Ag when expressed in a T cell (Gross et al., 1989). This receptor endowed the T cells with an “antibody like specificity.” Since this initial report, a number of modifications have been made to CARs to improve their signalling ability. The original first generation CARs consisted of an scFv linked to a CD3ζ chain which, upon phosphorylation of the ITAMs within the chain, could mediate downstream signalling. However, although this provided “signal one” (stimulation of receptor by specific Ag), the “signal two” (co-stimulation) was absent, which resulted in T cell

anergy. To overcome this problem, second generation CARs were developed that incorporated the CD28 co-stimulatory molecule. CD28 is the best studied of T cell co-stimulatory molecules and provides a second T cell stimulation signal by interacting with the B7 molecules CD80 and CD86 on the surface of APCs. The second generation CARs have proved more successful than the first generation receptors and can enhance Ag specific T cell activation as evidenced by increased cytokine secretion and T cell proliferation. Furthermore, third generation CARs have been established, which incorporate an additional co-stimulatory domain within the second generation complex.

One of the main advantages of CARs is that they are not restricted by MHC and therefore can be used universally irrespective of a patients MHC haplotype. Furthermore, CARs can be derived to target any protein in the body that a mAb can be raised to ensuring a broader range of specificities than a native TCR. Additionally, CARs should be able to function in any T cell subset as co-receptor interaction with MHC would be redundant. However, CARs are restricted to extracellular expressed Ags whereas gene therapy using TCRs has the added benefit of targeting internal Ags that are expressed in the context of MHC on the cell surface.

1.6.4. The Use of T Cells With Re-directed Ag specificity in The Clinic

In 1999 the first study was published that used retroviral mediated gene transfer of an MHCI restricted TCR to redirect the Ag specificity of human T cells (Clay et al., 1999). Since this study, the use of engineered T cells in cancer immunotherapy has grown rapidly. In particular, the group of Dr Steven Rosenberg have performed a number of clinical trials using TCR gene engineered T cells in the treatment of melanoma. The first of these studies used a γ-retroviral vector to transfer a HLA-A2 restricted TCR specific for the melanoma Ag MART-1 to isolated peripheral blood lymphocytes. This clinical study identified that the TCR gene engineered T cells were well tolerated in all patients and in 2/15 (13%) patients treated, the engineered T cells mediated regression of large established tumours. In a second

study, the same group engineered T cells to express a TCR with a higher reactivity towards MART-1, with the hypothesis that cells bearing this TCR would mediate greater cancer regression (Johnson et al., 2009). However, these T cells had on target reactivity towards basal level MART-1 expression in normal melanocytes in the skin, eyes and ears. Despite these on target toxicities, clinical responses were seen in 6/20 (30%) patients treated with high avidity MART-1 specific T cells. Interestingly, this high avidity MHCI restricted TCR was co-receptor independent and could redirect the Ag specificity of $CD4^+$ T cells as well as $CD8^+$ T cells. In the same study, T cells were engineered to express a high avidity gp100 specific TCR that was cloned from a HLA-A*02-01 transgenic mouse immunised with the human gp100 Ag. The particular gp100 epitope is not conserved between mice and humans and therefore central tolerance mechanisms do not prevent the release of high avidity murine α-human gp100 specific T cells from the thymus. In total sixteen patients were treated with murine TCR expressing T cells and 19% showed cancer regression with tumours responding in the lung, brain, lymph nodes liver and spleen. These studies therefore identified the ability of TCR engineered T cells to traffic to sites of Ag expression. Clinical trials have also been performed using T cells re-directed towards the NY-ESO-1 Ag, which is expressed in many tumours but expression in healthy tissue is restricted to cells in the testis (Robbins et al., 2011). The HLA*02-01 restricted TCR used contained two amino acid substitutions in the CDR3 region of the TCRα chain, which increased the affinity of the TCR for cognate pMHC enabling re-direction of both $CD4^+$ and $CD8^+$ T cells. An objective clinical response was seen in 4/6 synovial cell carcinoma patients and 5/11 melanoma patients treated with NY-ESO-1 specific gene engineered T cells (Robbins et al., 2011). Furthermore, despite the use of an affinity modified TCR no on or off target toxicities by the engineered cells was observed. Although in this study TCRs with increased affinity showed no toxicity, this is not always the case. A phase I clinical trial administered T cells engineered to express an affinity enhanced TCR specific for an HLA*01-01 restricted epitope of MAGE 3, which resulted in the death of the two treated patients a few days after infusion due to cardiogenic shock (Cameron et al., 2013; Linette et al., 2013). It was later identified

that the affinity enhanced T cells could kill *in vitro* generated cardiomyocytes due to reactivity towards the protein titin found in striated muscle. Despite extensive pre-clinical testing of this TCR the cross reactivity was not predicted, highlighting a need for further examination of the affinity enhancement of TCRs for gene therapy.

Clinical trials have also been reported using T cells engineered to express first generation CARs. In one study, T cells expressing a first generation CAR targeting the α-folate receptor failed to show efficacy in the treatment of ovarian cancer, which was potentially due to poor persistence (< 1 month) of the engineered T cells (Kershaw et al., 2006). Another study assessed the use of T cells expressing CARs specific for the tumour associated Ag CAIX in renal cancers, showed no clinical response observed on target liver toxicity. A severe case of on target toxicity resulted in the fatality of a metastatic adenocarcinoma patient who was treated with T cells expressing a third generation CAR specific for ERBB2 (Morgan et al., 2010). It was reported that low-level expression of ERBB2 in the lung resulted in a cytokine storm leading to patient death. The most successful clinical outcomes have been observed in the treatment of B cell leukaemia using T cells expressing CARs directed to the B cell specific Ag CD19. In a trial published in 2012, eight patients with advanced B cell malignancies were treated with CD19 T cells and IL-2 with six patients obtaining clinical remission (Kochenderfer et al., 2012). A similar clinical trial assessed the *in vivo* persistence and expansion of CD19 CARs with and without a CD28 signalling domain and identified an enhanced persistence and expansion of the CD19 CAR with both CD3ζ and CD28 signalling domains (Savoldo et al., 2011). Additionally, a third study identified that CD19 CARs incorporating CD3ζ and a CD137 signalling domain could mediate cancer regression and persist *in vivo* for up to 6 months (Porter et al., 2011). These studies identified the requirement of TCR activation and co-stimulation in the *in vivo* persistence and efficacy of adoptively transferred T cells.

The success of T cell engineering in cancer immunotherapy has provided the basis for investigating the use of TCR gene engineered T_{REG} cells in mediating tolerance

to transplants and in the treatment of autoimmune disease. In particular, the studies on viral vector design, T cell persistence *in vivo* and the TCR affinity and avidity requirements to ensure efficacy but prevent toxicity. In a study by *Tsang et al*, murine T_{REG} cells with indirect allospecificity (recognition of donor MHC presented by host APCs) were generated by retroviral TCR gene transfer and found to mediate tolerance to partially mis-matched heart transplants (Tsang et al., 2008). This study showed that co-transfer of a short-term immunosuppressant with TCR transduced T_{REG} cells promoted a significant increase in allograft survival compared to the use of immunosuppression alone. Another study by *Wright et al* revealed the therapeutic potential of TCR engineered T_{REG} cells in the treatment of a mouse model of rheumatoid arthritis (Wright et al., 2009). Using retroviral vectors the TCR genes for an A^b MHCII restricted ovalbumin TCR was transferred into αCD3/CD28 stimulated murine T_{REG} cells. The Ag inducible model of rheumatoid arthritis used, involved the immunisation of mice with methylated BSA followed by an intra-articular knee re-challenge with the same Ag. To assess the therapeutic potential of ovalbumin specific T cells, the mice were re-challenged with a methylated BSA and ovalbumin mix in one knee and methylated BSA alone in the other. The TCR transduced T_{REG} cells were then adoptively transferred into the mice and found to selectively home to and decrease inflammation in the ovalbumin challenged knee. This data suggested that Ag specific T_{REG} cells could home to the site of Ag expression and mediate local immune suppression of pathogenic T cells. A later study by *Brusko et al* identified that the Ag specificity of human T_{REG} cells could be successfully re-directed using LV mediated TCR gene transfer (Brusko et al., 2010). Unconventionally, this study used a MHCI restricted TCR, which was specific for the melanoma Ag tyrosinase, to re-direct the specificity of human T_{REG} cells. First, this study identified that neither the phenotype, expansion potential or suppressive capabilities of human T_{REG} cells were affected by gene transfer using LV vectors. Secondly, the study identified that T_{REG} cells expressing an MHCI restricted TCR could directly recognise tissue bearing cognate pMHCI. A tumour model system was set up whereby HLA-A2 transgenic mice were transplanted with an HLA-A2 tyrosinase-expressing tumour. In this model, adoptively transferred

TCR transduced T_{REG} cell could prevent the tumour pathogenesis mediated by transferred T_{EFF} cells expressing the same TCR. These studies have identified a means to generate therapeutic numbers of Ag specific T_{REG} cells. It is therefore possible that T_{REG} cells re-directed towards a disease relevant Ag specificity may be beneficial in the treatment of autoimmune disease.

1.7. Aims of this project

The aim of this thesis is to test the hypothesis that the LV mediated transfer of MHCI restricted TCRs can re-direct the Ag specificity of human T_{REG} cells towards MHCI restricted islet Ags. As discussed, the adoptive transfer of polyclonal T_{REG} cells is currently being investigated as a treatment for patients with T1D. However, there is strong evidence from animal models that Ag specific T_{REG} cells would persist for longer *in vivo*, home to the sites of Ag expression and be more efficacious at suppressing autoimmunity than their polyclonal counterparts (Tang et al., 2004; Tarbell et al., 2004). Furthermore, an increased potency of these cells would allow for a reduced quantity of Ag specific T_{REG} cells required for transfer. Conventional $CD4^+$ T_{REG} cells possess TCRs that are restricted towards MHCII self-Ags; however, the expression of MHCII in the inflamed islets of patients with T1D is minimal, whereas MHCI is hyper-expressed (Itoh et al., 1993; Willcox et al., 2009). Therefore, it is hypothesised that T_{REG} cells re-directed towards an MHCI islet Ag would favour trafficking to the site of Ag presentation within the islets and mediate Ag specific suppression at the site of inflammation upon recognition of pMHCI complexes on the surface of the islets.

LV gene transfer of TCR genes provides an elegant solution to generating large numbers of T_{REG} cells directed towards a defined Ag specificity. Currently, a strong rationale for the use of engineered T cells has been provided in the field of cancer immunotherapy, opening the possibility that this technique could be applied in other clinical settings including autoimmune disease. Additionally, protocols have already been thoroughly investigated in the ideal design of viral vectors for TCR

transfer. Furthermore, the report by *Brusko et al,* and a more recent report have provided a pretext for engineering human $CD4^+$ T_{REG} cells to express an MHCI restricted TCR (Brusko et al., 2010; Plesa et al., 2012). However, neither of these studies used an autoimmune disease relevant MHCI restricted TCR. Therefore, this thesis will detail a proof of principle study in the feasibility of transferring a TCR restricted to an MHCI restricted autoimmune disease relevant epitope to human $CD4^+$ T_{REG} cells.

The main areas that this thesis will investigate are:

1. The expression and pairing of TCRα and TCRβ chains on the surface of model cells lines post LV gene transfer as well as the ability of MHCI TCRs to re-direct the Ag specificity in the absence of the CD8 co-receptor.
2. The ability of MHCI TCRs to confer Ag specific suppressive capabilities to human $CD4^+$ T_{REG} cells.
3. Methods to optimize the expression and function of MHCI restricted TCRs in $CD4^+$ T_{REG} cells.

2. Materials and Methods

2.1. Culture of Cell lines and Primary Cells

Cell Lines

Human Embryonic Kidney 293T (HEK 293T) cells were maintained in 10mls of DMEM + Glutamax (Gibco, Life Technologies, UK) supplemented with 10% foetal calf serum (FCS) (Gibco, Life Technologies, UK) and 1% Pen/Strep (D10F) in $10cm^2$ tissue culture dishes (Corning, UK). The cells were maintained by splitting at a 1:3 ratio every two days using TrypLE express (Gibco, Life Technologies, UK) as a dissociation agent. Jurkat 76 (J76) cells, a TCRαβ deficient Jurkat cell line, were used for verification of MHCI restricted TCRs (Heemskerk et al., 2003). These cells transfected with the human CD8α chain (J76CD8α) were a kind gift from Professor Woolfgang Uckert (Max-Delbrück-Centre, Berlin, Germany). J76 and J76CD8α cells were cultured in RPMI + Glutamax (Gibco, Life Technologies, UK) supplemented with 10% FCS and 1% Pen/Strep/Fungizone (R10F) and maintained by splitting at a 1:5 ratio every 2–3 days.

Blood Donors

For initial studies, blood was obtained with informed consent from individuals who are therapeutically bled owing to primary heamochromatosis, but who are otherwise healthy. All blood donors were genotyped for HLA-A2*01 expression prior to their blood being used for experiments in this study and each individual subject denoted with an HAE number. For further experiments, leukapheresis cones were purchased from the National Blood Transfusion Service (Tooting, London, UK). Cells from these healthy control donors are denoted with a T number in this thesis. Staining with an anti-HLA-A2 antibody prior to use assessed the HLA-A2 positivity of these blood donors. All experiments in this study were conducted with ethical approval under the heading “Development of protocols for the generation in vitro of clinical grade T cells for adoptive cell therapy for use in autoimmune, inflammatory and malignant diseases” (REC reference 09/H0707/86).

Isolation of PBMCs

PBMCs were isolated by density centrifugation. Briefly, 30mls of undiluted blood was layered onto 15mls of lymphoprep (Axis Shield, Scotland) and centrifuged at 1000g for 20 minutes (Heraeus Multifuge 1, Thermo Scientific, USA). The interphase layer was collected into 50ml falcon tubes (Corning, UK) and washed in warm PBS (Gibco, Life Technologies, UK) at 300g for 10 minutes. The PBMC pellet was re-suspended in 20mls PBS and a second centrifugation step of 200g for 10 minutes was performed. The cell pellet was again re-suspended in 20mls PBS and viable cells were enumerated on a haemocytometer using trypan blue (Sigma-Aldrich, UK) for dead cell exclusion. The cells were then either cryopreserved or stained with fluorescently labelled antibodies for cell sorting by FACS.

Cryopreservation and Recovery of Cryopreserved Cells

Cells to be cryopreserved were pelleted by centrifugation at 4°C using a standard centrifugation step of 5 minutes at 400g. The supernatant was removed, the pellet re-suspended in 500 μl FCS per 10^7 cells and the cells placed on ice. An equal volume of FCS + 20% DMSO (Sigma-Aldrich, UK) was added, drop wise to the cell suspension with gentle agitation, to bring the final percentage of DMSO to 10%. The cells were added to 2ml cryovials (Corning, UK) in a total volume of 1ml, placed in a CoolCell (BioCision, USA) and transferred to the -80°C freezer. For long-term storage, the cells were transferred to liquid nitrogen 24 hours after cryopreservation. Cells were recovered by incubation in a 37°C water bath until a small ice pellet remained, followed by drop wise addition of cell culture media. The cells were then centrifuged at 400g for 5 minutes and re-suspended in the appropriate growth media.

Flow Cytometry Surface Staining

For FACS and flow cytometry, cells were harvested into round bottom polystyrene FACS tubes (BD, UK) and washed in at least 2mls of FACS buffer; PBS supplemented with 2% FCS and 0.5mM EDTA. The supernatant was decanted and

a master mix of fluorescently labelled antibodies was added to the residual buffer. The cells were stained on ice for 25 minutes with the addition of 0.25μl 7AAD (Calbiochem, Merck Millipore, UK) for the final 5 minutes and then washed in at least 2 mls of FACS buffer. Flow cytometry samples were acquired on a FACS Canto II (BD, UK) using FACS Diva software v6.0 (BD, UK). For cell sorting by FACS, cells were stained in sterile polypropylene FACS tubes (BD, UK) and sorted on a FACS Aria III (BD, UK). All Flow cytometry data was analysed using FlowJo software v9.4.11 (TreeStar, USA). All antibodies were titrated before use to ensure the highest discrimination between negative and positive populations. A full list of all antibodies used in this study can be found in Table 2-1.

HLA-A2 staining of whole blood

To determine the HLA-A2 status of blood donors, 10μl of whole blood was aliquoted into two FACS tubes and diluted at a ratio of 1:10 in PBS. Either 2μl of an anti HLA-A2 antibody or 2μl of an isotype-matched control antibody (IgG2b) was added to each tube, vortexed and incubated at room temperature, around 22°C, for 15 minutes in the dark. Red blood cell lysis buffer (BD, UK) was diluted at a ratio of 1:10 in ddH20 (MiiliQ, Merck Millipore, UK) and 2mls were added to each FACS tube. The FACS tubes were again vortexed and incubated for 15 minutes at room temperature, in the dark. Each tube was then washed twice in 2mls of PBS and the cells were immediately acquired on the FACS Canto II.

Flow Cytometry Staining for intracellular FoxP3

For intracellular FoxP3 staining, the FoxP3 transcription buffer staining kit (Ebioscience, UK) was used and the one step protocol for intracellular (nuclear) proteins followed. To minimise any activation induced up-regulation of FoxP3 on expanded and transduced populations, cells were rested in IL-2 free media overnight. Following cell surface staining, the cells were washed, vortexed to dissociate the pellet and re-suspended in 1 ml fix/perm buffer for 1 hour at room temperature in the dark. The cells were then washed twice in perm buffer, the supernatant removed and 20μl FoxP3-APC antibody added. The cells were stained

in the dark for 1 hour at room temperature in the dark, washed twice and immediately acquired on the FACS Canto II.

Table 2-1 List of Antibodies

Target	Conjugate	Clone	Origin	Dilution	Manufacturer
CD3	PE Cy7	HIt3a	Mouse	1/100	Biolegend
CD4	APC	SK3	Mouse	1/100	Biolegend
CD8α	FITC/APC-Cy7	RPAT8	Mouse	1/100	Biolegend
CD14	FITC	HCD14	Mouse	1/20	Biolegend
CD19	FITC	3G8	Mouse	1/50	Biolegend
CD25	PE	M-A251	Mouse	1/5	BD
CD45 RA	eflour450	HI100	Mouse	1/20	Ebioscience
CD69	APC	FN50	Mouse	1/5	Biolegend
CD127	PerCP Cy5.5	ebioRDR5	Mouse	1/20	Ebioscience
FoxP3	APC	259D	Mouse	1/20	BD
HLA-A2	FITC	BB7.2	Mouse	1/50	BD
IgG2b κ	FITC	27-35	Mouse	1/50	BD
RatCD2	PE/FITC	OX34	Mouse	1/400	Biolegend
TCRvβ2	PE	MPB2D5	Mouse	1/10	Beckman
TCRvβ5a	FITC	1C1	Mouse	1/20	Thermo
TCRvβ8	PE	56C5.2	Mouse	1/10	Beckman
TCRαβ	PE	IP26	Mouse	1/80	Biolegend

Multimer Staining

The HLA-A2 biotinylated monomers containing the preproinsulin $(PPI)_{15\text{-}24}$ epitope ALWGPDPAAAA (HLA-A2 ALW) and $GAD65_{114\text{-}122}$ epitope VMNILLQYV (HLA-A2 VMN) were a kind gift from Garry Dolton (Cardiff University, UK). Monomers were thawed on ice and tetramers were assembled by x 5 additions of streptavidin-APC (Life Technologies, UK) at 20 minute intervals on ice. PBS was added to each tube

to give a final working volume of tetramer of 0.2μg/ml. HLA-A2 SL9 dextramer (Immunodex, Copenhagen, Denmark) was used as per manufacturer's instructions. All multimers were centrifuged at 10000g for 2 minutes and only the supernatant was used for staining. Between $2x10^5$ and $5x10^5$ cells were aliquoted into FACS tubes and washed in PBS. The supernatant was decanted, leaving a residual volume of 80μl to which 40μl 15nM protein kinase inhibitor (PKI) solution (Dasatinib, Axon Medchem, Netherlands) was added. The tubes were incubated for 30 minutes at 37°C prior to addition of 0.5μg multimer to each tube and incubated for a further 10 minutes at 37°C. The cells were then washed in FACS buffer and surface stained with CD3 antibody and 7AAD as previously described. The cells were kept on ice until analysis to prevent multimer degradation.

Isolation and Activation of Primary T cell Populations.

PBMCs were stained with fluorescently labelled antibodies against CD4, CD25, CD127 and CD8 and sorted into three cell populations, $CD8^+$ T cells, $CD4^+CD25^-CD127^+$ effector T cells (T_{EFF}) and $CD4^+CD25^+CD127^-$ regulatory T cells (T_{REG}). The cells were also stained with CD14, CD16 and CD56 FITC conjugated antibodies for exclusion on this channel of monocytes and NK cells. Positively selected cells were sorted into FACS tubes containing 2mls of X-VIVO 15 (Lonza, Switzerland) 10% heat inactivated Human AB serum (GE healthcare, UK) (XV10) and centrifuged at 400g for 10 minutes. Established protocols were followed for the activation of cells (Brusko et al., 2010; Canavan et al., 2012; Skowera et al., 2008). Cells were activated using αCD3αCD28-coated magnetic beads (Dynal, Life Technologies, UK) at a bead to cell ratio of 1:1 for both $CD8^+$ T cells and $CD4^+$ T_{REG} and 0.5:1 for $CD4^+$ T_{EFF}. The culture media used was X-VIVO 5% Human AB (XV5) supplemented with 200IU/ml IL-2 (Proleukin, Novartis, UK) for $CD8^+$ T cell growth, 50IU/ml IL-2 for T_{EFF} cell growth and 600IU/ml IL-2 for T_{REG} cell activation. All cells were activated in a 96-ubottom plate (Corning, UK) at 50,000 cells per well.

Preparation of Feeders

Irradiated PBMCs were used to support expansion of transduced $CD8^+$ T cells. Cryopreserved PBMCs from three donors, at least one an HLA-A2*01 donor, were thawed and re-suspended in XV10 at a cell concentration of $5x10^6$ ml. The cells were then irradiated with 3000 RAD. Irradiated cells were then washed three times with XV5 and re-suspended in fresh XV5 at the desired cell ratio.

Cell Labelling with Cell Tracker Dye

For FACS-based proliferation and Jurkat cell activation assays, target cells were labelled with Cell Trace Violet Stain (Life Technologies, UK). Briefly, cells to be labelled were washed twice in PBS to remove all traces of serum. A 1uM working stock of Cell Trace Violet was prepared by dilution of 2μl of 5mM stock concentration in 10mls of PBS. A total of 1ml of violet working stock was added per 10^7 cells to be labelled. For equal labelling, cells were gently agitated for 10 minutes in the dark at room temperature before quenching of the reaction with an equal volume of FCS for 2 minutes. The cells were then washed in RPMI, 20% FCS by centrifugation at 400g for 5 minutes before use in assays.

Statistical Analysis

An unpaired Student's t Test was used to calculate the statistical significance of peptide specific stimulation of transduced T cells compared to non-Ag specific stimulation where data was normally distributed. Where indicated, a paired Student's t Test was used to calculate the statistical significance of endogenous TCR expression and introduced TCR expression within the same cell population. All means, standard deviations and statistical significance were calculated using GraphPad Prism5 software.

2.2. Molecular Biology Techniques and Cloning

2.2.1. General Molecular Biology Methods

Plasmids pMDG-2 Vesicular Stomatitis Virus-G (VSV-G) expressing plasmid and P8.91 expressing *gag* and *pol* for viral encapsulation were a kind gift from the group of Michael Malim (Kings College London, UK). The 1E6 and 868 TCRα and TCRβ gene were inserted into the pELNS third generation SIN LV vector (Richardson et al., 2008) and were kindly provided for this study by John Bridgeman (Cardiff University, UK). The generation of all other plasmids used for this study is described in subsequent sections.

Restriction Digest

All restriction enzymes used in this project were purchased from NEB and included XbaI, SalI, EcoRI and NsiI. For a standard 10μl restriction digest of plasmids, the following reaction was set up: 1μl of 10x buffer, 1 unit of enzyme (as defined by NEB), 1μg of plasmid DNA and molecular biology grade H_2O (Ambion, Life Technologies, UK) to 10μl. This mixture was then vortexed, the contents collected by brief centrifugation and incubated for 1 hour in a 37°C water bath. The reaction mix was then loaded on to a 1% agarose gel for visualisation of the final product. For preparations of inserts and plasmids for ligation reactions, restriction digests were scaled up to 50μl with all reagent ratios kept as standard.

Gel Electrophoresis and Extraction

Agarose gel electrophoresis was used for analysis of DNA fragments. 1% agarose gels with a 1:10,000 dilution of SyberSafe (Life Technologies, UK) were made by dissolving agarose powder (Sigma-Aldrich, UK) in 5% Tris-Borate-EDTA (TBE) buffer and used to resolve products of 0.7–7kb in size. For resolution of standard 10μl restriction digest reactions, 2μl of 6x blue loading dye (NEB, UK) was added to each tube. The 12μl reaction was subsequently loaded into the agarose gel and 5x TBE was used to cover the gel in the gel electrophoresis tank (Biorad, UK), The gels were run at 100V for 1 hour or until the products and ladder had fully

separated before being visualised using a UV transilluminator (BioRad, UK). For cloning, PCR products were excised from agarose gels using sterile razor blades and purified using the QIAquick Gel Extraction kit (Qiagen, UK) following the manufactures protocol. Briefly, the excised fragments were weighed, dissolved in three gel volumes of QG buffer before one gel volume of >99% molecular biology grade isopropanol (Sigma Aldrich, UK) was added. This mix was loaded and bound onto a QIAquick spin column, washed and eluted in 30μl of buffer EB. The DNA was quantified using a NanoDrop1000 spectrophotometer (LabTech, USA).

Ligation

All ligation reactions performed in this study utilised the LigaFast Rapid DNA Ligation System (Promega, UK). For standard cohesive end ligations a molar ratio of vector to insert of 3:1 was employed were the amount of vector used was 50ng. The amount of insert DNA required was calculated using the following formula: 50ng of vector x insert size (Kb)/ size of vector (Kb) x 1/3. In a 15μl reaction, 7.5μl of 2x rapid ligation buffer and 2μl of T4 DNA ligase were added to the volume of DNA calculated with sterile H_2O used to complete the 15μl total reaction volume. If preparations of vector or insert where too dilute then a Vacuum Concentrator (Eppendorf, Germany) was used to reduce the DNA to the required concentrations. Negative control reactions were included, whereby sterile H_2O was substituted for insert DNA. Each reaction was incubated for 1 hour at room temperature before being stored overnight at 4°C.

Transformation of Plasmid DNA

Plasmid DNA was transformed into chemically competent cells for mass production of the plasmid. For standard transformation of VSV-G, p8.91 and pELNS viral vectors, including ligation reactions using these vectors, XL-10 Gold Cells (Agilent, UK) were used. One shot Top10 competent cells (Life Technologies, UK) were used when cloning into the pGEM T Easy Vector (Promega, UK). As standard protocol, competent cells that were stored at -80°C were thawed on ice for 10 minutes. Either 50ng of plasmid DNA or 4μl of a ligation reaction were then added

to each vial of competent cells, mixed by flicking, and incubated on ice for 30 minutes. The bacteria were then subjected to heat shock at 42°C for 45 seconds and then placed on ice for 5 minutes. SOC media (Sigma-Aldrich, UK) that had been preheated to 42°C was added to each vial (900μl for XL-10 and 450μl for Top10 cells) and each vial incubated in a 37°C shaking incubator (New Brunswick Scientific, Eppendorf, Germany) for 2 hours. The transformed cells were then plated onto LB agar plates supplemented with 100μg/ml Ampicillin (Amp) (Sigma-Aldrich, UK) employing sterile technique; typically 100μl of cells were plated for each ligation reaction whereas 100μl of a 1:20 dilution of plasmid DNA was plated for standard transformations. The plates were left to air dry for 10 minutes followed by incubation at 30°C for 16–20 hours.

Preparation of DNA from Bacterial Colonies

Successful transformants were picked from the agar plates using a pipette tip that was subsequently used to both re-streak a second agar plate and inoculate 3 mls of LB broth 100μg/ml Amp. The re-streak plates were incubated at 30°C and the LB-Amp broth in a shaking incubator set to 30°C for 16–20 hours. Re-streak plates were wrapped in parafilm and transferred to 4°C for future use. The cultures were then centrifuged in 1.5ml Eppendorfs at 8000g for 5 minutes and the plasmid DNA extracted using the QIAprep Spin MiniPrep Kit (Qiagen, UK) following the manufacture's protocol. The DNA was eluted from the QIAprep Spin Columns in 30μl of elution buffer. Following this, the plasmid DNA was then used in a restriction digest reaction to check for successful ligation or correct plasmid preparation using diagnostic cuts and any unused plasmid stored at -20°C.

2.2.2. Clonotyping of the 1D7 TCR RNA Extraction

RNA was extracted from the 1D7 clone, a $CD8^+$ T cell clone specific for the $GAD65_{114\text{-}122}$ epitope, in order to determine the sequence of the TCR (Knight et al., 2014). Total RNA from this clone was extracted from previously cryopreserved

cells that had been stored in RLT buffer at -80°C using the RNeasy Mini Kit (Qiagen, UK) according to manufacturer's protocol.

cDNA Conversion

In order to fully elucidate the complete sequence of the unknown TRAC and TRBC genes of the 1D7 clone, the SMARTer RACE cDNA Amplification kit (Clontech, France) was used. The specifics of this technique are described in Figure 2-1 and the manufacturer's protocol was adhered to. For initial first strand synthesis 3.25μl of RNA, or as a negative control ddH_2O, was incubated with the 5' CDS Primer A in 0.2μl PCR tubes (Appleton Woods, UK) and heated to 72°C for 3 minutes and then cooled to 42°C for 2 minutes using a 2720 Thermo Cycler (Applied Biosystems, Life Technologies, UK). To each tube, 6.25μl of a previously prepared mastermix incorporating 2μl 5x buffer, 1μl DTT, 1μl DNTP, 0.25μl RNase inhibitor, 1μl SMART reverse transcriptase enzyme and 1μl of the SMARTer oligo, was added. This mixture was then incubated at 42°C for 120 minutes and the reaction terminated by heating to 72°C for 10 minutes. Each tube was then washed out using Tricine-EDTA (TE) (Sigma-Aldrich, UK) at a 1:1 ratio prior to the purification of the reaction using the Nucleospin Gel and PCR Clean up kit (Machery Nagel, Germany)

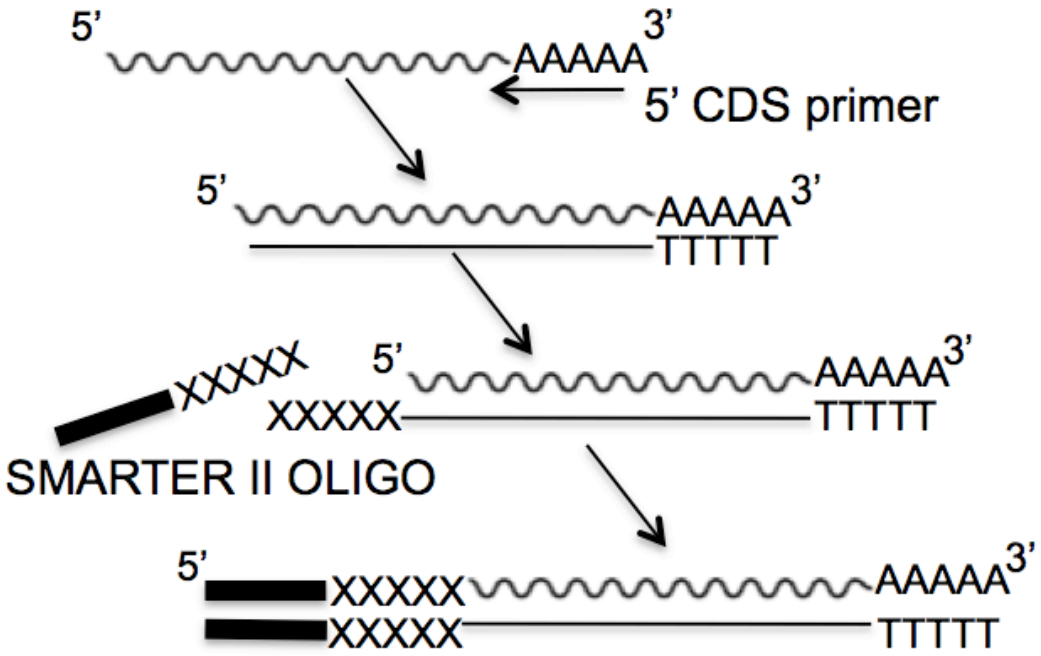

Figure 2-1 SMARTer 5' RACE cDNA Synthesis. All polyadenylated RNA within the extracted RNA was amplified using the 5'CDS primer, the sequence of which is 5' T(25)XXX where X are nucleotides other that T. This step ensures that complete mRNA strands will be amplified from the 3' end. The SMARTScribe Reverse Transcriptase (RT) enzyme exhibits terminal nucleotide transferase, which enables the addition of extra nucleotides to the 3' end of the transcribed mRNA. The SMARTer II Oligo is able to bind the extra nucleotides and the RT enzyme is able to switch template from the mRNA molecule to the SMARTer II oligo. The RT then transcribes 5' to 3' generating a complete cDNA molecule from the original mRNA, incorporating the SMARTer sequence.

PCR Amplification of 1D7 TRAC and TRBC Gene

The 1D7 cDNA incorporating the SMARTer RACE sequence was PCR amplified using the universal primer A mix (UPM) (Clontech, France) and primers specific for either the C region of the TRAC gene (CCAGGCCACAGCACT GTTGCTCTTGAAGTCC) or the TRBC gene (GCTGACCCCACTGTGCACCTCC TTCCC). The PCR reaction is described in Table 2-2 and an additional negative control was included for the PCR reaction with sterile H_2O. The PCR cycling reaction was initiated with a high temperature for 5 cycles, termed touchdown PCR, to allow the long UP to bind to the cDNA. This temperature was then reduced

for a second annealing step to enable the short UP to bind the DNA. The cycling reactions are detailed in Table 2-3. The 50μl reaction was then purified using gel electrophoresis as described with the expected PCR product size for both the TCRα and TCRβ chains being between 500–700bp in size (Figure 2-2 (A)). The negative controls from the initial cDNA conversion and PCR reaction were run on separate gels to confirm absence of DNA.

Table 2-2 PCR Reaction Mix for the Amplification of the 1D7 TRAC and TRBC Genes

Reagent	Volume (μl)
cDNA	5
5x Phusion	10
2000U/ml	0.5
0.2μM dNTP	1
0.2μM	1
1x UPM	5
Molecular	27.5
Total	50

Table 2-3 PCR Cycling condition for the Amplification of the 1D7 TRAC and TRBC Genes

PCR Condition	Temperature (°C)	Time (s)	Cycles
Initial Denature	98	30	1
Denature (1)	98	10	5
Annealing (1)	70	30	5
Elongation (1)	72	40	5
Denature (2)	98	10	30
Annealing (2)	65	30	30
Elongation	72	40	30
Final Elongation	72	60	1

Poly-A-Tailing and Ligation into the pGEM T vector

For sequencing of PCR products, the PCR products were poly-A-tailed and ligated into the pGEM T Easy vector system (Promega, UK). For poly-A-tailing, the 30μl gel elution was concentrated to a volume of 8μl using a vacuum concentrator (Eppendorf, Germany). The 8μl gel elution was then incubated with 1.14μl Taq, 1.14μl 10x advantage 2 PCR buffer (Clontech, France), 0.456μl dATP (Life

Technologies, UK) and 0.664μl H_2O. This mixture was incubated in a 70°C heat block (Jencons PLS, UK) for 30 minutes, with centrifugation after 15 minutes to collect any contents that may have evaporated. The ligation reaction was then completed using 4μl of poly-A-tailed insert and 1μl of the pGEM T easy vector or 4μl sterile H_2O as a negative control.

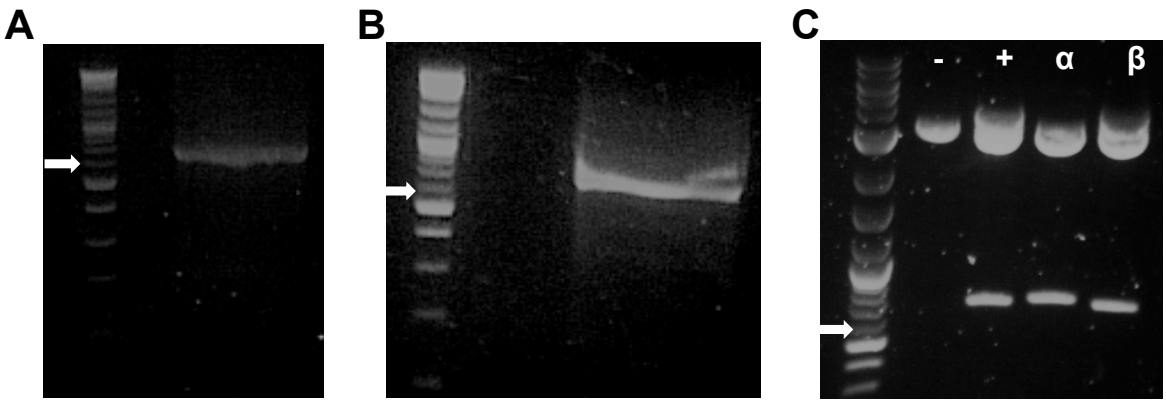

Figure 2-2 Clonotyping of the 1D7 TCR. TCRα and β genes were identified from the 1D7 clone using SMARTer 5' RACE technology. Agarose gels show the resolved PCR products of (A) 1D7 cDNA with the incorporated 5' SMARTer RACE sequence, amplified with a TCRα constant region primer (TRAC) and Universal Primer Mix (UPM) A (B) and the 1D7 cDNA amplified with a TCRβ constant region primer (TRBC) and UPM. (C) The 1D7 TCRα and TCRβ PCR products were ligated into the pGEM T easy vector and transformed into TOP10 competent cells, from which DNA was prepared. EcoRI restriction sites flank the cloning site of the pGEM T easy vector and an agarose gel shows successful ligation of a TCRα and TCRβ chain into the vector, which were sent for sequencing. The – control represents an empty vector ligation and the + control was a digest of a previously successful PCR product ligation. The furthest left hand lane was loaded with a 2-log DNA ladder (NEB, UK). The white arrows in each gel picture indicate the 600bp marker,

Sequencing of the TCR chains

The ligations of the 1D7 α and β chains into the pGEM T easy vector were transformed into TOP10 cells and the DNA prepared as described. A single colony was also picked from the negative control plate. A restriction digest reaction of each plasmid using EcoRI, which has two cut sites in the pGEM T easy vector flanking the T overhang sites, was preformed (Figure 2-2 (C)). The plasmid preparations that were positive for a 500–700bp insert after the restriction digest

were considered successful ligation products and at least 3 DNA preparations for each chain were shipped to MWG Eurofins, Germany, for DNA sequencing. The DNA was sequenced using the T7 promoter, which is 52bp 5' of the first T overhang. Upon receipt, the DNA sequences were imputed into the IMGT website (www.IMGT.org) to ascertain the variable regions of the TCRα and TCRβ chains. Using Geneious Pro 5.6.3 software (Biomatters Ltd, Auckland) the full sequencing of the 1D7 TCRα and TCRβ chains were constructed (Figure 2-3).

1D7 TCRα

MMKSLRVLLVILWLQLSWVWSQQKEVEQNSGPLSVPEGAIASLNCTYSDRGSQS
FFWYRQYSGKSPELIMFIYSNGDKEDGRFTAQLNKASQYVSLLIRDSQPSDSATY
LCAVTGSYIPTFGRGTSLIVHPYIQNPDPAVYQLRDSKSSDKSVCLFTDFDSQTNV
SQSKDSDVYITDKTVLDMRSMDFKSNSAVAWSNKSDFACANAFNNSIIPEDTFFP
SPESSCDVKLVEKSFETDTNLNFQNLSVIGFRILLLKVAGFNLLMTLRLWSS

1D7 TCRβ

MLLLLLLLGPGSGLGAVVSQHPSRVICKSGTSVKIECRSLDFQATTMFWYRQFPK
QSLMLMATSNEGSKATYEQGVEKDKFLINHASLTLSTLTVTSAHPEDSSFYICSAS
YSGSNEQFFGPGTRLSVLEDLKNVFPPEVAVFEPSEAEISHTQKATLVCLATGFY
PDHVELSWWVNGKEVHSGVSTDPQPLKEQPALNDSRYCLSSRLRVSATFWQNP
RNHFRCQVQFYGLSENDEWTQDRAKPVTQIVSAEAWGRADCGFTSESYQQGVL
SATILYEILLGKATLYAVLVSALVLMAMVKRKDSRG

Figure 2-3 Amino Acid Sequence of the 1D7 TCRα and TCRβ Chains. The amino acid sequence of the 1D7 TCRα is as shown with the signal peptide highlighted in blue, the variable region in red and the TCRα constant region in black. The 1D7 TCRβ sequence highlights the signal peptide and variable region in blue and red respectively followed by the sequence of the TCRβ constant region 2.

Slight modifications were made in the addition of an extra disulphide bond between the TCRα and TCRβ chains (Cohen et al., 2007) and appropriate restriction sites at both the 5' and 3' end as shown (Figure 2-4). This complete gene was then synthesised by MWG Eurofins, which included codon optimisation of the entire sequence. Upon receipt of the fully synthesised gene it was subjected to restriction digest with XbaI and NsiI and ligated into a prepared pELNS.1E6.RatCD2 vector

that had been similarly digested to removed the 1E6 α and β TCR genes. This process resulted in the generation of the pELNS.1D7.RatCD2 construct.

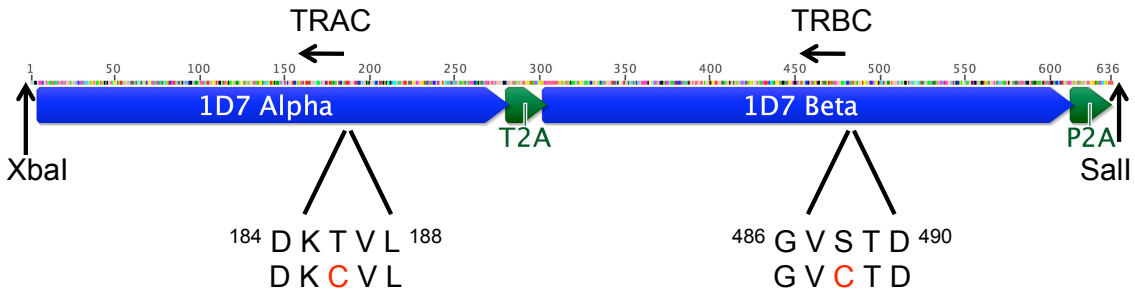

Figure 2-4 1D7α.T2A.1D7β.P2A Gene Synthesis. The black arrows show the position of the TRAC and TRBC primer sites. Owing to these positions the 3' end of the 1D7 α and β chain constant regions were absent from the original sequence. To rectify this, the 1D7 TCRα and TCRβ chains were aligned with the sequences of the 1E6 counterpart chains to complete the full TCR chains. Additionally, a T2A and P2A cleavage sequence were included before and after the 1D7 β chain. The restriction sites XbaI and SalI were similarly included at the positions shown. Finally, the amino acid substitutions shown in red were preformed to include a second disulphide bond between the 1D7 TCRα and TCRβ chains to improve pairing as previously described.

2.3. Lentivirus Production

Plasmid Maxi-Preparations

For production of lentivirus, plasmids were prepared using the Nucleobond Endotoxin Free Maxiprep kit (Machery Nagel, Germany). Briefly, a single colony was picked from a transformation plate and used to inoculate a 3ml starter culture of LB–Amp, which was incubated for 6–8 hours at 30°C in a shaking incubator. An overnight culture of 600 ml LB–Amp was inoculated with 600μl of starter culture and incubated at 30°C for 16–18 hours with shaking. The bacteria were pelleted at 6000g for 10 minutes re-suspended in re-suspension buffer, lysed and then the lysis reaction neutralised according to manufacturer's protocol. The lysate was then loaded onto an equilibrated NucleoBond Xtra Column, which allows simultaneous loading of the column and clearance of lysed bacteria through a column filter. After two wash steps to ensure complete recovery of endotoxin-free

plasmid, the plasmid was eluted in 15mls of elution buffer. For DNA precipitation, 10.5mls of molecular grade isopropanol (Sigma-Aldrich, UK) was added drop wise to the eluted DNA, vortexed and centrifuged at 15,000G for 30 minutes at 4°C. The DNA pellet was then washed in 70% ethanol, left for 20 minutes at room temperature to air dry and re-suspended in TE buffer (Machery Nagel, Germany). The yield of DNA was assessed by a NanoDrop1000 spectrophotometer and the plasmid DNA concentration adjusted to 1μg/ml.

Transfection

293T cells were seeded at a density of $1x10^6$/ml in a six-well plate 24 hours prior to transfection in 2 ml D10F. For each well to be transfected 1μg of p8.91, 1μg of genome vector and 0.5μg of VSV-G were incubated with 498.5μl of serum free, DMEM. To a separate tube, 10μg of polyethylenimine (PEI) (Polysciences, Germany) was mixed with 490μl serum free DMEM. Both were incubated for 5 minutes before mixing together and incubating for 20 minutes at room temperature. 1 ml of D10F was removed from the 293T cells and replaced with 1 ml of the transfection mixture, which was added drop wise to the 293T cells. The media on the transfected cells was changed after 6 hours and the LV containing supernatant was collected 48 and 72 hours post transfection. Supernatant collected at 48 hours was filtered through a 0.45μm filter (Minisart, Sartorious, Surrey) and stored at 4°C overnight before being pooled with the clarified 72 hour supernatant. Typically, 8 x 6 well plates were transfected for each LV preparation.

Lentivirus Concentration

LV was dispensed into 38.5 ml ultracentrifuge tubes (Beckman Coulter, UK) and was pelleted by centrifugation at 28K RPM for 1 hour and 15 minutes in an ultracentrifuge (Sorval, Thermo Scientific UK). The supernatant was decanted and 100μl of serum-free DMEM added to each pellet. The ultracentrifuge tubes were kept on ice for 1 hour to aid the LV pellet re-suspension before the pellet was re-suspended by pipetting. The virus was then topped up with DMEM for a final virus

concentration of 100x. The LV was then divided into 100μl aliquots and stored at -80°C.

Lentivirus Titration

293Ts were plated in a 24 well plate overnight at a density of $1x10^5$/ml/well. The next day the LV to be titrated was thawed and 5 x 1:2 serial dilutions were preformed in a total volume of 500μl D10F. The medium on the 293Ts was removed and replaced with the diluted LV. The 293Ts were spin infected at 1200g for 1.5 hours at room temperature. The media on the 293Ts was replaced with D10F and transduction assessed 72 hours later by flow cytometry. The titre of the LV was calculated by (% of cells transduced * total number of cells) / volume of LV in mls.

Transduction of Jurkat Cells and Generation of Cell Lines.

Jurkat cells were harvested, counted and re-suspended to a cell ratio of $1x10^6$/ml in R10F. 1ml of cells was aliquoted into 1.5ml sterile eppendorfs (Appleton Woods, UK) per transduction and centrifuged at 8000g for 5 minutes. The cell pellet was re-suspended in LV supernatant at 5TU/ml and the volume made up to 100μl. The tubes were incubated for 2 hours at room temperature with agitation every 15 minutes. The Jurkat cells were spin infected for 45 minutes at 1000g in a micro centrifuge (Eppendorf, Germany). The cell pellet was re-suspended in R10F and cultured for 72 hours before assessment of transduction by flow cytometry. Transduced cells were then FACS sorted based on TCRαβ expression and cultured for use in subsequent assays.

Primary Cell Transduction

Primary cell populations were sorted and activated for 48 hours as described in section 2.2. The cells were harvested, counted and $2x10^5$ cells were added to 1.5 ml sterile eppendorfs. The cells were then centrifuged in a micro centrifuge at 8000g for 5 minutes and the cell pellet re-suspended in LV supernatant at 5TU/cell. The primary cells were then transduced using the same protocol for Jurkat

transduction. After the transduction period, the LV supernatant was removed and replaced with the specific cell culture media for each cell population and added to a well of a 96 flat bottom plate.

3. Evaluating the Transfer of MHCI Restricted TCRs in Model T cell Lines

3.1. Introduction

The experiments in this chapter were designed to fully test the expression and function of the MHCI restricted TCRs that are used throughout this thesis. These three HLA-A2*01 restricted TCRs, summarised in Table 3-1, are namely the 868 TCR, the 1E6 TCR and the 1D7 TCR. Historically there was thought to be a strong association with the HLA-A2*01 allele and susceptibility to T1D, with one report in children with T1D showing a frequency of 60-70% of this allele (Fennessy et al., 1994). Since then, more comprehensive genome wide association studies have identified other MHCI alleles that have a stronger association with susceptibility to T1D than the HLA-A2*01 allele (Howson et al., 2009; Nejentsev et al., 2007). However, HLA-A2*01 is known to be the most predominant Class I HLA-A allele – genotyping of 5 US populations has revealed that 49.8% of the American Caucasian population carry this allele (Ellis et al., 2000). Therefore, re-direction of human $CD4^+$ T_{REG} cell with HLA-$A2^*01$ restricted antigenic relevant autoreactive TCRs could potentially be a tailored treatment for a large percentage of the population.

Table 3-1 Variable Chain usage of TCR's

TCR	TRA	TRB	TRBV MAb	Index Peptide
868	TRAV12-2*01	TRBV5-6*01	TRBv5	HIV-1 $P17_{77-85}$
1E6	TRAV12-3*01	TRBV12-4*01	TRBv8	PPI_{15-24}
1D7	TRAV12-2*01	TRBV20-1*02	TRBv2	$GAD65_{114-122}$

868 TCR

The 868 $CD8^+$ T cell line was first expanded using the immunodominant HIV GAG peptide SL9 (SLYNTAVTL) in 1996 from an HIV^+ subject known as patient

868 (Sewell et al., 1997). This cytotoxic T lymphocyte (CTL) line was found to successfully lyse autologous B cell targets that were pulsed with the SL9 peptide. After attempts to isolate a single clone from this 868 $CD8^+$ T cell line by limiting dilution were unsuccessful, clones were isolated using HLA-A2-SL9 phage display. These clones were then used to generate TCR bearing phage, from which the TCR genes of each clone could be sequenced. One of the clones sequenced was found to have a TCR made up of a TCRβ chain from the TRBV5-6 family and a TRAV12.2 family TCRα chain (IMGT nomenclature) (Varela-Rohena et al., 2008). This confirmed previous findings in the 868 patient whereby isolated SL9 MHCI $tetramer^+$ T cells were predominately $TRBV5\text{-}6^+$ (Wilson et al., 1998). Additionally, further analysis of the 868 CTL line revealed that all HLA-A2-SL9 $tetramer^+$ cells expressed a TCR composed of the TCRBV5-6 and TRAV12.2 chains. To further characterise this TCR, now known as the 868 TCR, a soluble recombinant form was generated. Surface plasmon resonance (SPR) could therefore be used to measure the affinity for which the 868 TCR binds the SL9 peptide. Interestingly, this TCR-pMHCI interaction was identified as the highest affinity interaction of its kind so far on record (dissociation constant Kd of 85nM as measured by kinetic injection analysis), which was in fact 25-fold higher than any other TCR-pMHC interaction documented (Cole et al., 2007a). The identification of this high affinity interaction led to this TCR being selected as an ideal candidate to investigate the re-direction of the Ag specificity of human primary T cells using MHCI TCRs (Plesa et al., 2012). In fact, the 868 TCR was found to not only be able to re-direct the Ag specificity of human $CD8^+$ T cells but also of human $CD4^+$ T_{EFF} and $CD4^+$ T_{REG} cells. These findings therefore identified the interaction between the 868 TCR and cognate SL9 peptide as a CD8 independent interaction. Therefore, this TCR will serve as a useful positive control for the transfer of MHCI TCRs into $CD4^+$ T_{REG} cells.

1E6 TCR

The parental 1E6 $CD8^+$ T cell clone was cloned in 2008 from a patient with T1D and recognises the preproinsulin $(PPI)_{15\text{-}24}$ epitope (Skowera et al., 2008). In this

first paper, the authors identified the PPI_{15-24} epitope, from herein designated ALW, as a naturally processed and presented HLA-A2 restricted Ag. To do this, the authors engineered a K562 cell line, an HLA negative chronic myelogenous leukemic cell line, to express both HLA-A2 and the human PPI genes. By engineering this cell line to encode the full PPI gene, they ensured that only PPI peptides that were naturally processed by the cells' peptide processing machinery could be loaded into the HLA-A2 binding groove. Therefore, upon acid elution of peptides from the HLA-A2 binding groove, the investigators were confident that the ALW peptide was a bona fide, naturally processed and present epitope (NPPE). Using MHCI tetramers, $CD8^+$ T cells with specificity for the ALW peptide were identified in the circulation of patients with T1D, proving the disease relevance of this peptide (Skowera et al., 2008; Velthuis et al., 2010). Following on from these data, the investigators isolated $CD8^+$ T cell clones specific for this peptide, which culminated in the identification of the 1E6 $CD8^+$ T cell clone. This clone was generated by the incubation of $CD8^+$ T cells from a T1D patient with ALW peptide pulsed autologous mature DCs (mDCs). After a second round of re-stimulation, cells were stained with an ALW HLA-A2 tetramer and positive cells were single cell sorted into 96 well plates containing allogeneic PBMCs, PHA and a cocktail of cytokines. Once a clone had been fully established the authors verified its specificity by tetramer staining and its ability to produce the pro-inflammatory cytokines TNF-α and IFN-γ upon peptide specific stimulation. Importantly, the 1E6 $CD8^+$ T cell clone was found to specifically kill β cells from an HLA-$A2^+$ donor highlighting the relevance of this clone, and subsequently its TCR *in vivo*. Since the initial identification of this clone, further work has been performed to characterise the clone and specifically it's TCR. One such study by *Bulek et al* assessed the structural basis behind the 1E6 $CD8^+$ T cell clones' autoreactivity (Bulek et al., 2012). In this study, it was identified that the 1E6 TCR's interaction with ALW-HLA-A2 was very weak, with a dissociation constant of only ~278μM Kd. This affinity is considered very low in comparison to published binding affinities for pathogen-specific TCR interactions with pMHCI, which are typically 1-10μM (Cole et al., 2007a). Despite this proven low affinity,

the 1E6 TCR is currently the only autoreactive disease relevant, MHCI restricted TCR that has been described and thus had been chosen as a candidate TCR for this study.

1D7 TCR

The 1D7 clone was isolated from a healthy control donor against the $GAD65_{114-122}$ epitope and was generated using the same protocol as for the 1E6 CD8 T cell clone (Knight et al., 2014). Upon incubation of this clone with human HLA-$A2^+$ islets, the clone was found to produce the inflammatory cytokine MIP-1β. Furthermore, when HLA-$A2^+$ K562 target cells that had been transfected with GAD65, were used to stimulate the clone it produced inflammatory cytokines such as IFN-α and IL-12 (Knight et al., 2014). At present no other work has continued to characterise the significance of this TCR, however the disease relevance of the HLA-A2 restricted $GAD65_{114-122}$ epitope has been shown in patients with T1D (Monti et al., 2008a; Monti et al., 2007). Thus, this TCR, which is specific for a peptide with evidence of disease importance, was chosen as a second autoreactive TCR for this study.

In order to initially validate these TCR constructs the Jurkat J76 clone was used as a model CD4 T cell line. This Jurkat cell line had previously been engineered to lack expression of both TCRα and TCRβ chains and thus serves as a useful tool in which the TCR expression constructs could be tested (Heemskerk et al., 2003). Moreover, the aim of this study was to ascertain whether the MHCI restricted autoreactive TCRs used in this project could effectively signal in the absence of the CD8 co-receptor. To this end the J76CD8α cell line, which was generated by transfection of J76 cells with CD8α, were also obtained (Schub et al., 2009). These two Jurkat T cell lines enabled the testing of both the signalling and CD8 dependency of the TCRs used in this project.

3.2. Material and Methods

Generation of pELNS.RatCD2 Mock Vector

The Rat CD2 gene was amplified from the pELNS.1E6.RatCD2 vector and using the following primers the XbaI restriction site was incorporated into the 5' end of the gene: forward primer (AAAATCTAGAATGCATATGCGGTGCAAG) and reverse primer (TTAGTCGACCCGCTTCTTC). The PCR reaction mix used was identical to those detailed in Table 2-2 and the PCR cycling reaction used is detailed in Table 3-2. The resulting PCR product was then purified using the Nucleospin Gel and PCR cleanup kit. To prepare the vector for which the Rat CD2 fragment would be inserted, the pELNS.1E6.RatCD2 vector was digested with XbaI and SalI, as previously described, in order to excise the 1E6 TCR chains and the residing Rat CD2 molecule. The restriction digest mix was run on a 1% agarose gel and the fragment excised and cleaned up using the QIAquick gel extraction kit. The concentration of DNA of the Rat CD2 and vector fragments was quantified using the NanoDrop and a 15μl ligation reaction was set up using a vector:insert molar ratio of 3:1, T4 DNA ligase and 2x rapid ligation buffer (Promega, UK). XL-10 cells were transformed using 4μl of the ligation mix. Ten successful transformants were picked, cultured and the plasmid DNA prepared. The DNA preparations were then screened by restriction digest with XbaI and SalI to visualise the correct 705bp sized Rat CD2 insert. The original pELNS.1E6.RatCD2 vector was used as a control and the digestion of this with XbaI and SalI yields a 2.6Kb fragment. This cloning strategy is summarised in Figure 3.1.

Table 3-2 PCR Cycling Conditions for Cloning of Rat CD2

PCR Condition	Temperature (°C)	Time (s)	Cycles
Initial Denature	98	30	1
Denature	98	10	25
Annealing	55	30	25
Elongation	72	40	25
Final Elongation	72	600	1

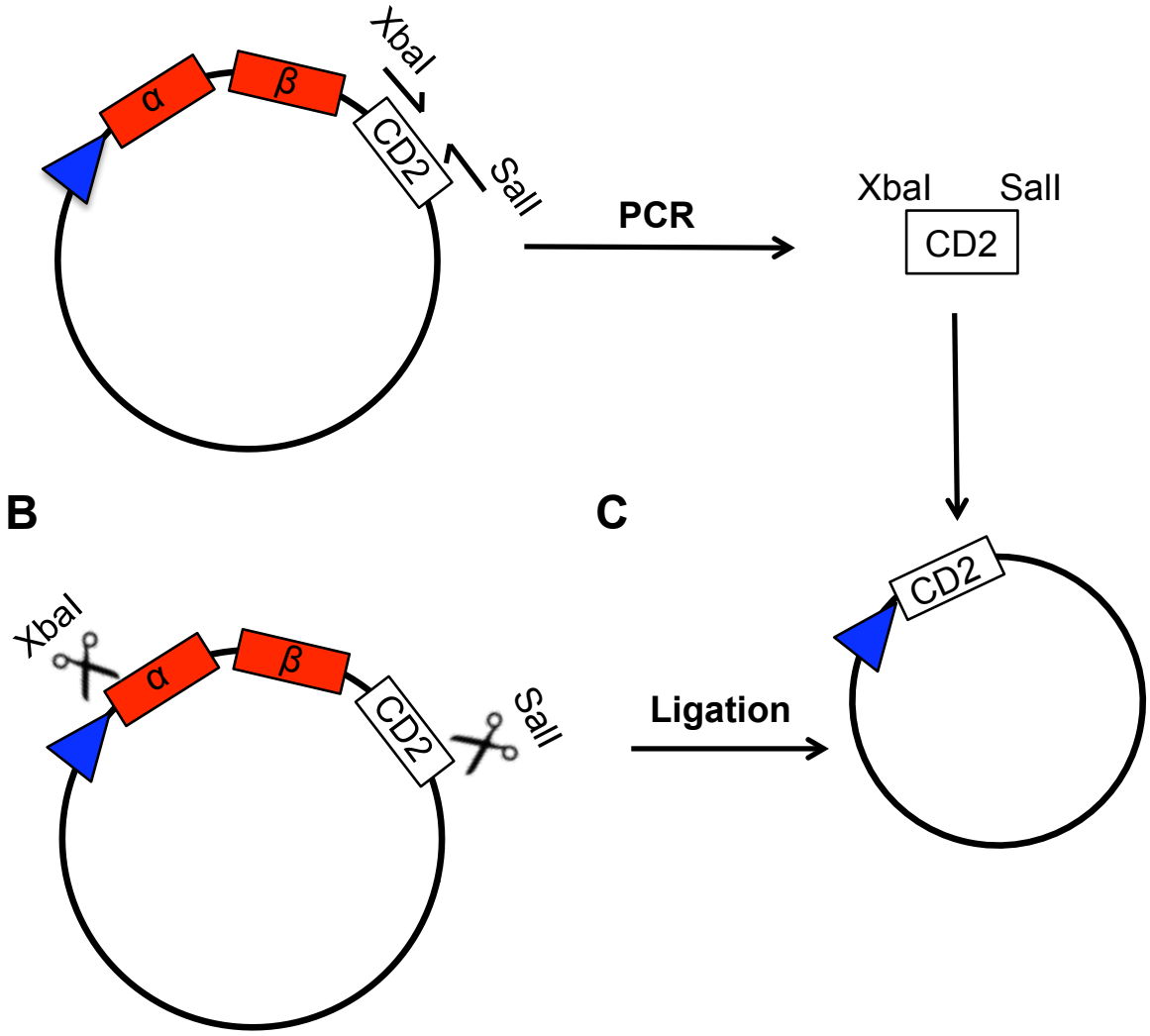

Figure 3-1 Cloning Strategy for Generation of Mock Vector. A Mock vector for the sole transcription of the Rat CD2 gene was generated as a control for LV transduction of cells. (A) The Rat CD2 gene was PCR amplified with 5'XbaI primer and 3'SalI primer using the pELNS.1E6.RatCD2 vector as a template. (B) Using restriction enzymes XbaI and SalI the pELNs.1E6.RatCD2 vector was digested to remove the full 1E6 TCR and Rat CD2 construct. (C) The PCR product from (A) and digested pELNS vector from (B) were ligated together to generate the pELNS.RatCD2 Mock vector.

Generation of pELNS.1E6.CD8α Vector

A CD8α gene fragment with flanking 5' NsiI and 3' SalI restriction sites was kindly provided by Professor Linda Wooldridge (University of Bristol). The pELNS.1E6.RatCD2 vector was then digested with NsiI and SalI restriction

enzymes to excise the Rat CD2 fragment and the remaining vector was purified. The vector and CD8α fragment were then ligated as described in Section 2.2.1. Ligations were subsequently transformed into competent cells, cultured overnight and DNA from the colonies prepared. To determine a successful ligation, prepared vectors were transfected into 293T cells and after 48 hours were stained with α-Rat CD2 and α-CD8α antibodies. A vector that yielded $CD8\alpha^{+}RatCD2^{-}$ 293T cells was then selected. A summary of this cloning strategy is detailed in Figure 3-2.

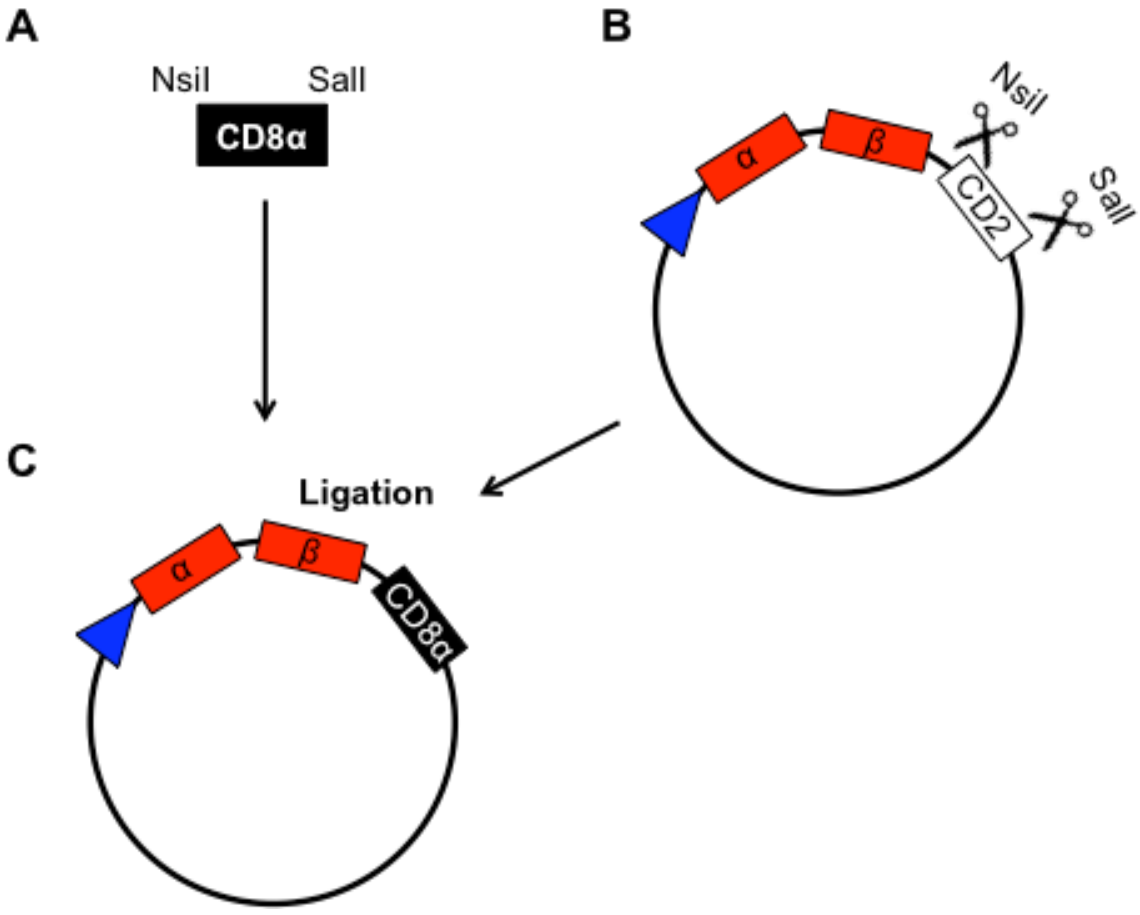

Figure 3-2 Cloning Strategy for the 1E6.CD8α Vector. For the generation of the pELNS.1E6.CD8α vector (A) a CD8α fragment with 5' NsiI and 5' SalI restriction sites was obtained. (B) The pELNS.1E6 vector was prepared by digestion with NsiI and SalI restriction sites to remove the Rat CD2 fragment. (C) The CD8α fragment from (A) was ligated into the prepared vector from (B) to generate the pELNS.1E6.CD8α vector.

Peptides

Peptides were obtained by custom peptide synthesis service (Thermo Scientific) to a purity of >95%. Peptide sequences were as follows **ALW** – ALWGPDPAAA, **RQF** – RQFGPDFPTI, **YQY** – YQYGPDFINA, **SL9** – SLYNTVATL, **VMN** – VMNILLQYV. All peptides used in this study, excluding SL9, were dissolved in PBS to a concentration of 1mg/ml. The SL9 peptide was dissolved in sterile DMSO to a concentration of 10μg/ml and diluted to 1μg/ml in PBS resulting in a final DMSO concentration of 10%.

Activation of Jurkat Cell Lines

Cryopreserved PBMCs from an HLA-A2*01^{+} donor were thawed and labelled with Cell Trace Violet as described in section 2-1. A schematic of the culture conditions for this assay are as shown (Figure 3-3(A)). Briefly, PBMCs were re-suspended to 4x10^{6}/ml and 1ml of cells was added to labelled 15ml falcon tubes. Peptide was added at a concentration of 10μg/ml, PBS and CytoStim served as negative and positive controls respectively. PBMCs were pulsed for 2 hours at 37°C with rotation and then washed in R10F at 400G for 5 minutes. All supernatant was removed from the pulsed PBMCs and the cells were re-suspended at a concentration of 1x10^{6}/ml. Jurkat cells to be activated were counted and re-suspended to 1x10^{5} cells/ml. For a final PBMC:Jurkat cell ratio of 10:1 an equal volume of cells was added to the wells of a 96-u bottom plate. The cells were incubated for 16 hours and the following day harvested into FACS tubes and stained with APC conjugated CD69 antibody and 7AAD. The gating strategy used for this assay is detailed (Figure 3-3(B)).

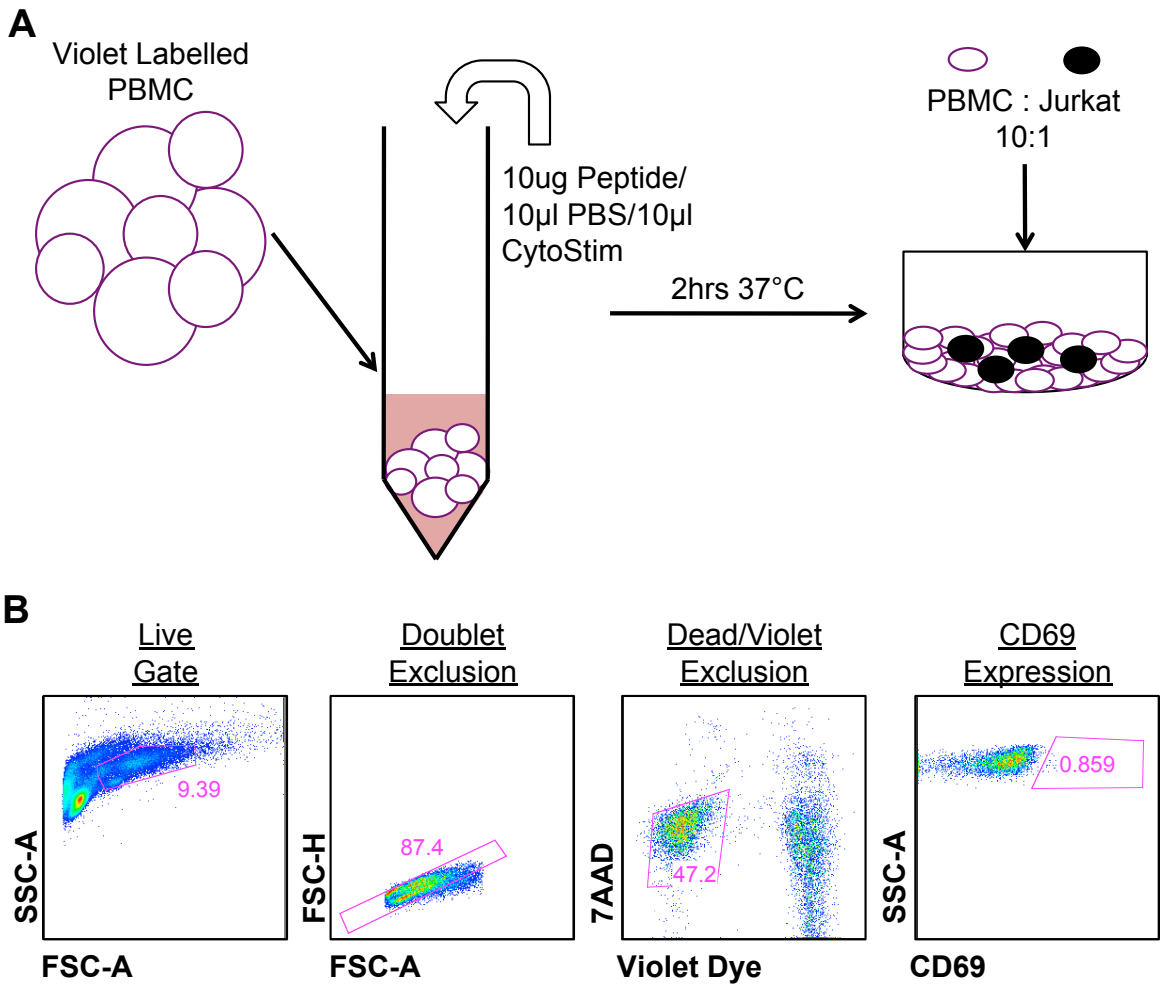

Figure 3-3 Schematic Representation of Jurkat Activation Assay. (A) Violet labelled PBMC were incubated in 1ml of R10F with the addition of 10µg of peptide, PBS or CytoStim for 2 hours at 37°C. The PBMCs were then washed and incubated with Jurkat cells at a ratio of 10:1 for 16 hours. (B) The assay was analysed by gating on Jurkat cells based on their FSC-A/SSC-A profile and excluding any doublets. Live Jurkat cells were then identified based on a $7AAD^{-}Violet^{-}$ phenotype and CD69 expression assessed on this population

3.3. Results

3.3.1. TCR constructs

For introduction of *de novo* TCRs into Jurkat cell lines both the pELNS.1E6 vector and the pELNS.868 vector were obtained from John Bridgeman (Cardiff University). Both the TCRα and TCRβ chains of each TCR had been clonotyped from the 1E6 and 868 parental clones previously. The pELNS LV vector is a third generation replication deficient vector that had initially been adapted from the pRRL-SIN-CMV-eGFP-WPRE vector (Richardson et al., 2008). The original adaptation made to the vector was to replace the cytomegalovirus promoter (CMV) promoter with the human elongation factor 1α promoter (EF1-α). Both promoters are considered strong promoters, however, the CMV promoter has been shown to be unreliable due to its silencing in certain mammalian cell lines (Qin et al., 2010), unlike the EF1-α promoter. The 1E6 and 868 TCRα and TCRβ chains were then inserted downstream of the EF1-α promoter and linked by a T2A linker site (Figure 3-4). The inclusion of the self-cleaving 2A peptide allows the generation of a multi-cistronic vector, which ensures equimolar transcription of all genes within the vector (Szymczak and Vignali, 2005). A further adaptation to the pELNS vector was to include a second 2A sequence downstream of the TCRβ chain followed by the Rat CD2 gene, which was a useful marker to assess transduction efficiency. The 1D7 TCRα and TCRβ chains were clonotyped and inserted into the pELNS vector as described in Chapter 2.2.3. Prior to insertion into the pELNS LV vector, all TCRα and TCRβ chains were codon optimised. Codon optimisation is a technique used to improve the translational efficiency of a gene by altering rare codons to more commonly used codons and can improve the expression of TCRαβ chains post transduction in primary cells (Scholten et al., 2006). To control for effects of LV transduction of cell lines a mock vector consisting of the EF1-α promoter followed by the Rat CD2 marker gene was generated (Figure 3-5 (A)). For construction of this control vector the 1E6 TCRα and TCRβ chains and Rat CD2 marker genes were excised from the pELNS vector using XbaI and SalI restriction enzymes (Figure 3-5 (B)). The Rat CD2

marker gene was then PCR amplified from the original pELNS vector to incorporate a 5' XbaI site allowing the PCR product to be ligated into the empty pELNS vector using sticky end ligation (Figure 3-5 (C)). Successful ligation products were analysed by restriction enzyme digest and agarose gel screen (Figure 3-5 (D)). The DNA from a single ligation product was subsequently prepared, verified and used as a control mock vector.

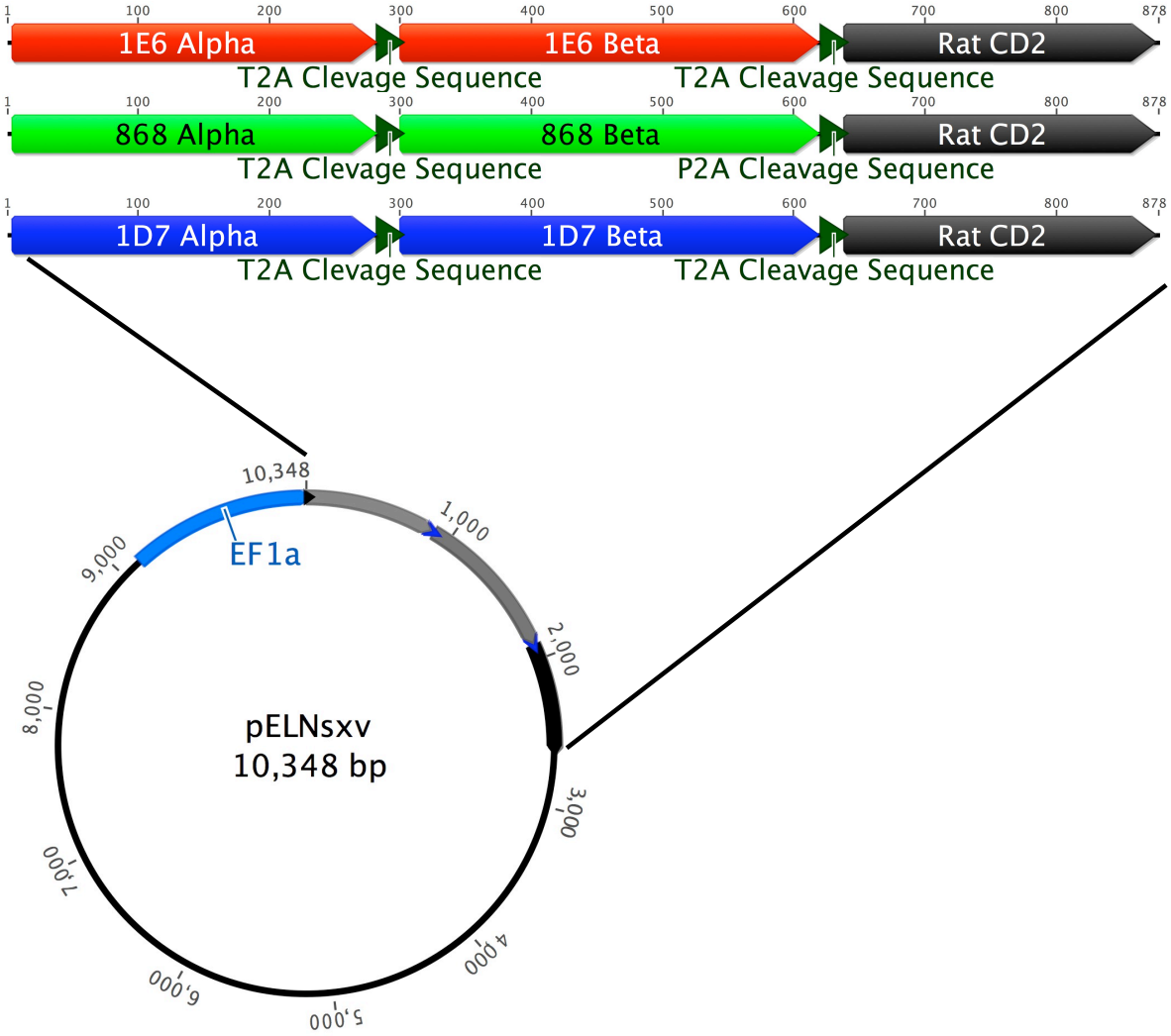

Figure 3-4 T-Cell Receptor Expression Plasmids. The TCR constructs, consisting of the α chain of the TCR linked by a T2A site of its paired TCRβ chain were inserted into the SIN pELNS lentivirus plasmid under the control of the EF1α promoter. A second cleavage site was inserted to link the Rat CD2 marker to the TCR chains, to assess for successful LV transduction.

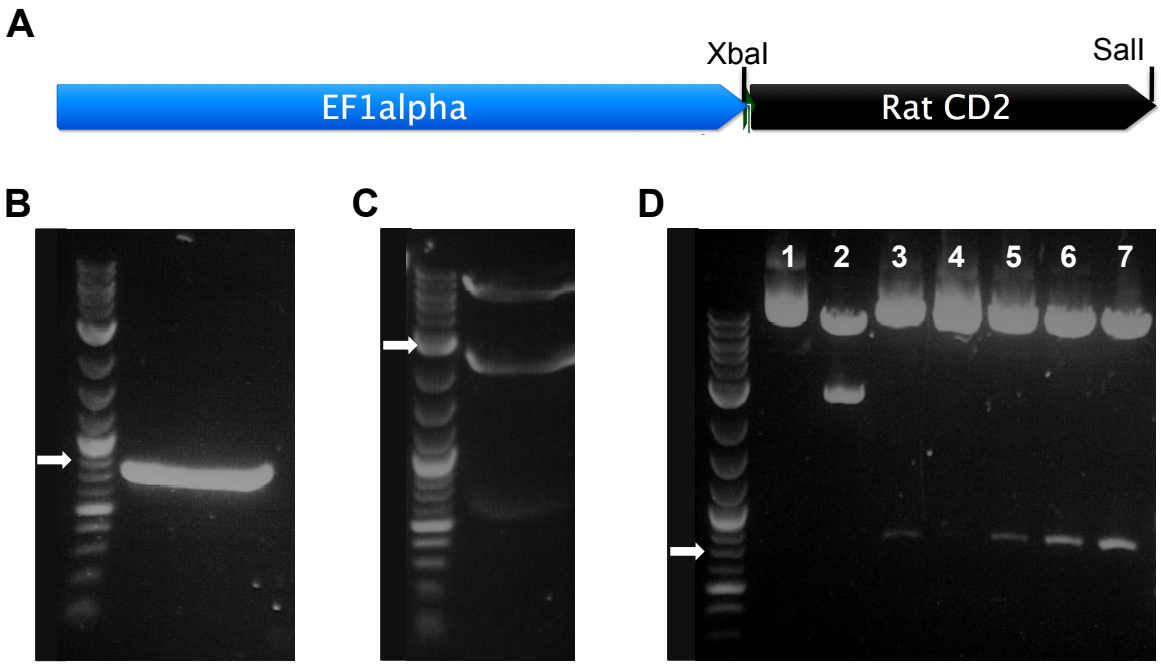

Figure 3-5 Generation of pELNS.RatCD2 Vector. (A) The pELNS.RatCD2 control mock vector consists of the Rat CD2 marker gene downstream of the EF1-α promoter. The Rat CD2 gene was PCR amplified from the pELNS.1E6.RatCD2 vector to incorporate a 5' XbaI site as indicated. (B) The 705bp product was resolved on an agarose gel as indicated by the white arrow and was excised and purified. (C) The pELNS.1E6.RatCD2 vector was digested with XbaI and SalI to remove the 1E6 TCR and Rat CD2 genes (sized 2.6Kb in total) and resolved on an agarose gel. The white arrow indicates the 3000bp marker (D) The DNA was prepared from bacteria transformed with the ligation products and digested with XbaI and SalI. Lane 1 shows the pELNs.1E6.RatCD2 un-digested. Lane 2 shows the pELNs.1E6.RatCD2 digestion product of a 2.6kb insert. Lanes 3-7 show multiple ligation preps whereby the insert is the 705bp fragment of Rat CD2 as indicated by the white arrow. The left hand lane of each gel was loaded with a 2-log DNA ladder.

3.3.2. Production and Titration of LV

The generation of LV for this project was performed by co-transfection of 293T cells with the pELNS transgene constructs, an envelope plasmid pMDG2-VSV-G (VSV-G) and a packaging plasmid p8.91, which is based on the HIV-1 LV with all dispensable genes for virus encapsulation removed as described (Zufferey et al., 1997) (Figure 3-6 (A)). Transfection efficiency of 293Ts was assessed by staining with an α-Rat CD2 antibody and was typically >75% for transfection with pELNS LV vectors (Figure 3-6 (B)). Post transfection, LV containing supernatant was harvested, concentrated 100x and titrated on 293T cells by spin infection of $2x10^5$ cells using 1:2 serial dilutions of LV. To calculate the titre in transducing units/ml, graphs were plotted of LV input against % transduction (Figure 3-6 (C)). Using the linear portion of the curve, the titre could be assessed using the calculation described in Chapter 2.4. Typically, the mock vector yielded higher titres than LV generated from TCR containing vectors (~$5x10^7$ TU/ml vs. ~$1x10^7$ TU/ml). However, the LV generated from each TCR containing vector yielded similar titres suggesting that the smaller mock vector is more efficiently packaged and thus produced greater numbers of virus particles compared to larger TCR containing vectors. The titres calculated in this manner were then used to calculate multiplicity of infection for transduction of Jurkat cell and primary cell populations in subsequent chapters (Figure 3-7).

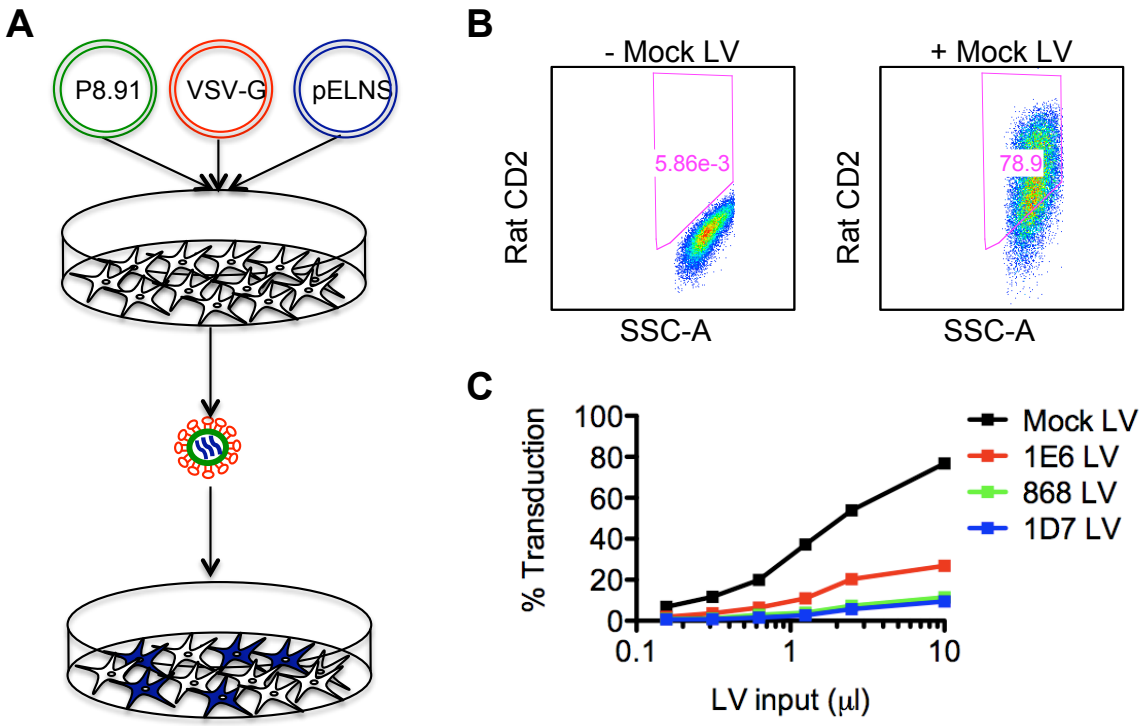

Figure 3-6 Production and Titration of LV. (A) LV was produced by co-transfection of 3 plasmids: VSV-G, p8.91 and pELNS transgene construct into 293Ts. The 293T cells produced packaged LV particles containing the transgene of choice, which was then validated by LV transduction of 293Ts. (B) 293Ts were transduced in the presence or absence of mock LV and then cultured for 72 hours. The successful transduction with mock LV was then assessed by staining with an α-Rat CD2 antibody. (C) The titre of each LV was calculated by transducing 293Ts with serial dilutions of virus. Titres were calculated by (% transduced cells * total cells transduced) / LV input.

3.3.3. Generation of TCR+ Jurkat T cell Lines

Jurkat T cell lines were generated by transduction of both the J76 and J76CD8α Jurkat T cell lines with LV encoding mock vector, the 868 TCR, the 1E6 TCR and the 1D7 TCR. Transduced cell were then sorted via FACS based on the expression of TCRαβ, for cells transduced with TCR encoding LV, or Rat CD2 for cells transduced with the control mock LV. Expression levels of TCRαβ, Rat CD2 and CD8α on sorted cell lines were assessed by flow cytometry following staining with the relevant fluorochrome-conjugated mAb (Figure 3-7 (A) and (B)). The mean fluorescent intensity (MFI) of TCRαβ staining for each transduced cell type was determined (Figure 3-7 (C)). For transduction of both the J76 and J76CD8α cells, the MFI of TCRαβ expression was greatest for the 868 TCR with MFI of 5126 and 8157 respectively. Expression of the 1E6 TCR in both Jurkat cell lines gave MFI readings of 3810 and 5540 for the $1E6^+$ J76 cells and $1E6^+$J76CD8α. The lowest TCR expressed was the 1D7 TCR with an MFI of 1821 in J76 cells and 2106 in J76CD8α cells. For all TCRs tested, the MFI expression in J76CD8α cells was higher than in J76 cells, as the J76CD8α cells have a slightly larger FSC-A/SSC-A profile. As expected, transduction with the mock vector gave negligible readings for TCRαβ MFI of 178 and 396 in the both cell lines. Within the transduced J76CD8α T cell population, the MFI of CD8α was consistent, showing that the difference in TCRαβ expression was TCR dependent and not cell line dependent (Figure 3-7 (C)). To ensure J76 cells were re-constituted with the correct TCR, each TCR cell lines was stained with the relevant TCR vβ specific antibody (Figure 3-8 (A-C)). Staining results revealed the correct vβ antibody staining for each TCR as detailed (Table 3-1). The vβ5 antibody stained the 868^+ J76 cells, vβ8 stained the $1E6^+$J76 cells and the 1D7 TCR stained with the vβ2 antibody. The mock J76 cells were negative for all antibodies tested.

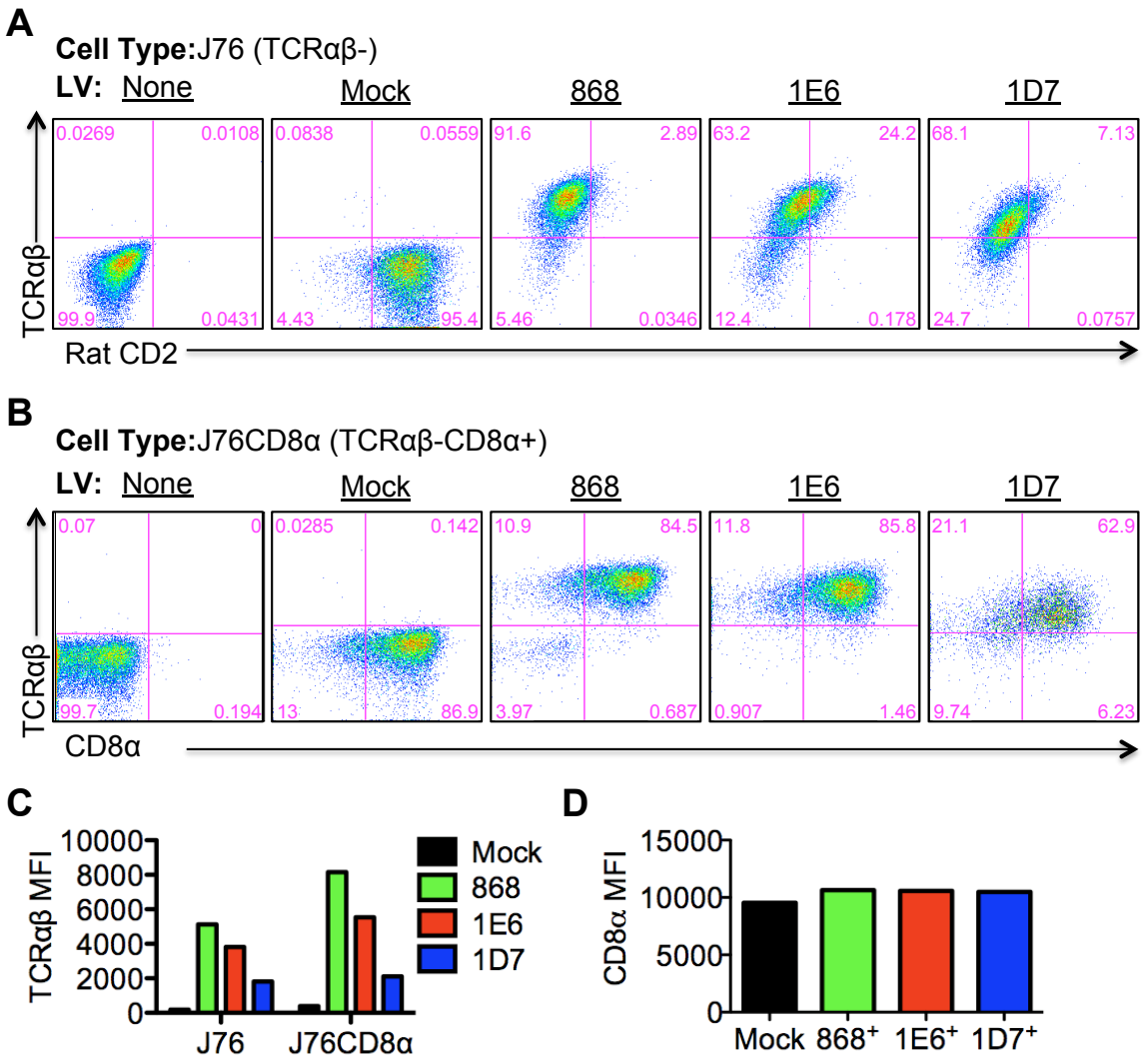

Figure 3-7 Transduction of Jurkat Cell Lines. TCR$^-$ Jurkat cell lines were transduced with 5TU/ml of the indicated LV (A) TCR$^-$ J76 cells and (B) TCR$^-$ CD8α$^+$ J76CD8α were stained with antibodies against TCRαβ, Rat CD2 and CD8α to measure successful expression of all constructs. Non transduced J76 cells served as a negative control for all antibodies tested. The mean fluorescence intensity of (C) TCRαβ expression on J76 and J76CD8α cells and (D) CD8α on transduced J76CD8α was calculated at the same time point on the transduced cell lines. Data is representative of staining on two individual occasions.

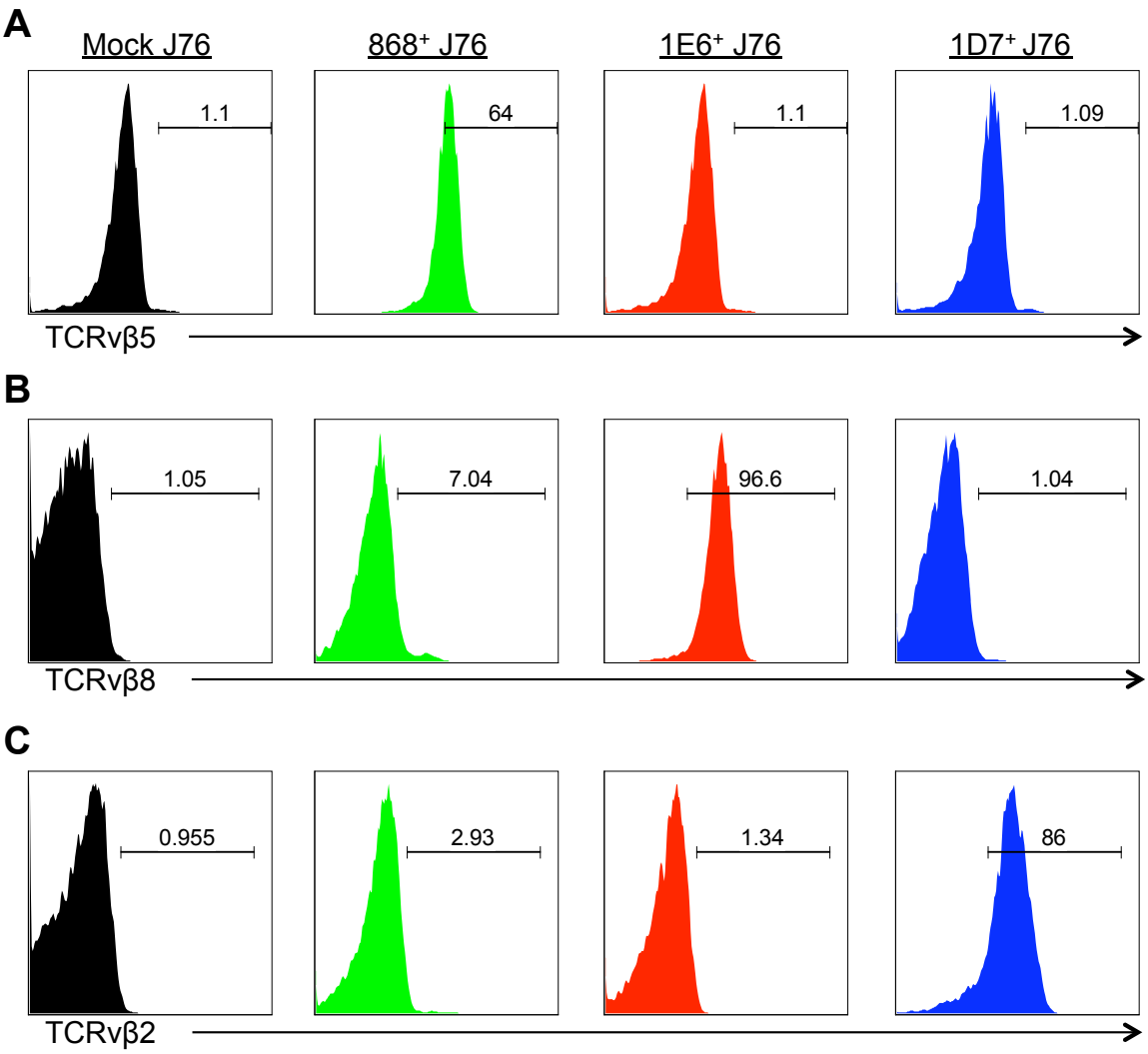

Figure 3-8 TCR transduced Jurkat cells express the specific vβ chain of each TCR. Transduced J76 cell lines were incubated with antibodies against (A) TCRvβ8, the 1E6 TCR vβ (B) TCRvβ5 of the 868 TCR and (C) TCRvβ2, which identifies the 1D7 TCR. Cells were gated on a live FSC-A/SSC-A gate and the positivity for each TCRvβ antibody was set to the 99th percentile based on Mock J76 cells staining, which were negative for each antibody tested.

3.3.4. Multimer Staining of Pathogen Specific and Autoreactive MHC Class I TCRs

MHCI multimers, such as tetramers and dextramers, are well-established reagents that can be used to identify Ag specific MHCI restricted TCRs (Wooldridge et al., 2009). Positive staining with a MHCI multimer therefore identifies a TCR that has the correct conformation of its TCRα and TCRβ chain, which enables it's binding to pMHC. Thus they are a useful tool to address that the TCR transduced Jurkat cell lines express a productive, correctly folded TCR$\alpha\beta$ as opposed to staining with monoclonal Abs that only infers expression of each chain. To this end, the ability of TCR transduced J76 and J76CD8α cells to stain with its Ag specific MHCI multimer was tested. Jurkat cell lines were similarly stained with a non-Ag specific, irrelevant multimer as a negative control. The high affinity 868 TCR transduced J76 and J76CD8α cell lines stained specifically with the SL9 MHCI multimer (Figure 3-9 (A) and (C)) but not with the irrelevant ALW MHCI multimer (Figure 3-9 (B) and (D)). The 1E6 and 1D7 transduced Jurkat cell lines were each stained with either the PPI specific ALW MHCI multimer or the GAD65 specific VMN MHCI multimer, with the non specific peptide for each TCR acting as the irrelevant multimer control. For the 1E6 TCR transduced Jurkat cell lines, it was interesting to note that only the 1E6$^+$J76CD8α, and not the 1E6$^+$J76 cell were capable of binding the ALW MHCI multimer (Figure 3-10). Moreover, only around 40% of CD3$^+$ 1E6$^+$J76CD8α cells were capable of binding ALW tetramer (Figure 3-10 (C)). The inability of the 1E6$^+$J76CD8α to stain with the VMN MHCI multimer demonstrated specificity as well as the correct TCR$\alpha\beta$ conformation of the introduced 1E6 TCR (Figure 3-10 (D)). The 1D7$^+$ Jurkat cells yielded a similar pattern of MHCI multimer binding to the 1E6$^+$ Jurkat cells in that only the 1D7$^+$J76CD8α, and not the 1D7$^+$J76 cells, demonstrated an ability to stain with the VMN MHCI multimer (Figure 3-11 (A and C)). This staining was above background as calculated by staining with the irrelevant ALW MHCI multimer (Figure 3-11 (D)). However, the positive VMN MHCI multimer staining was not as distinct as that seen when 1E6$^+$ or 868$^+$ cells were stained with their specific pMHC multimer. The proportion of cells that could

be identified with the VMN MHCI multimer was also minimal with less that 10% of cells showing positive staining.

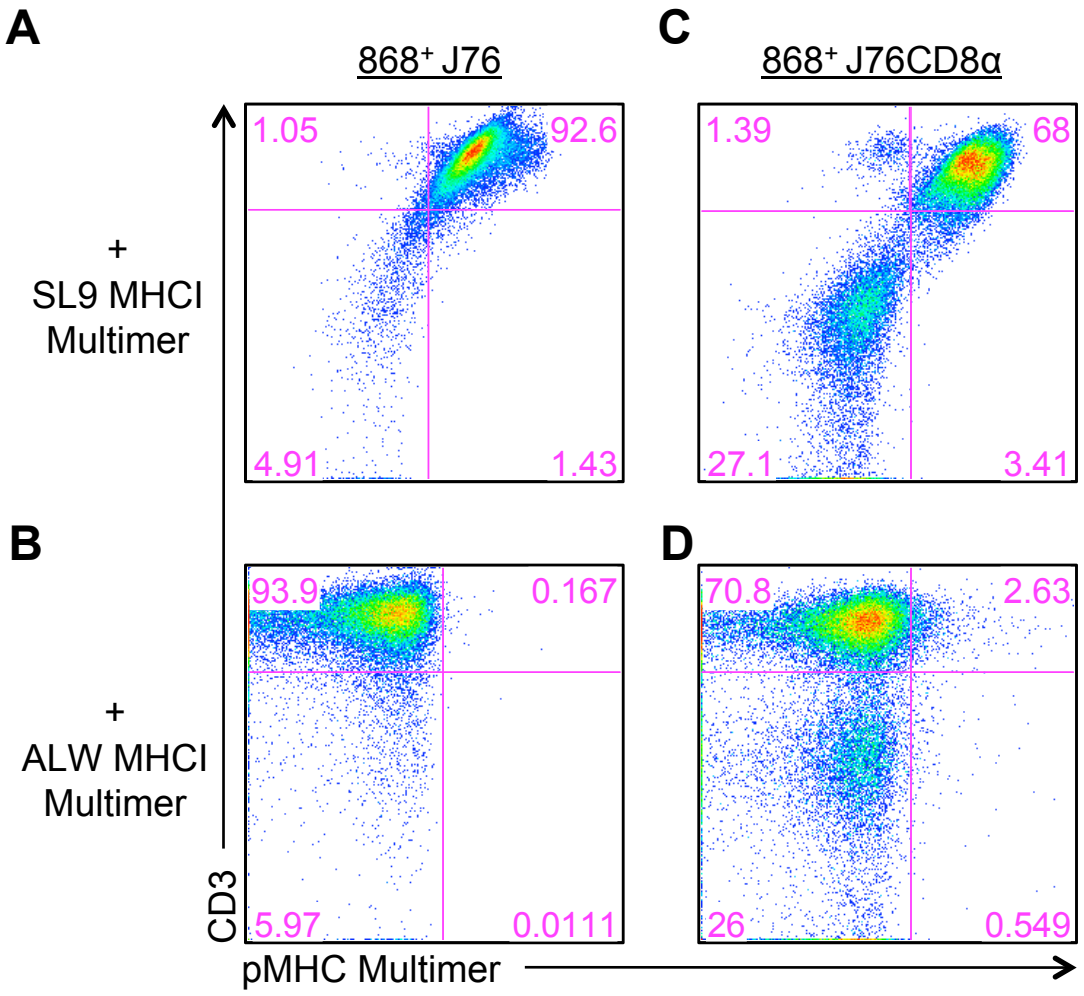

Figure 3-9 A SL9 MHCI Multimer efficiently and Specifically Identifies 868 TCR Transduced Jurkat Cell Lines. 868 transduced J76 cells were stained with either (A) peptide specific SL9 MHCI Multimer or (B) ALW MHCI Multimer as an irrelevant pMHC control. (C)-(D) J76CD8α cells were similarly stained.

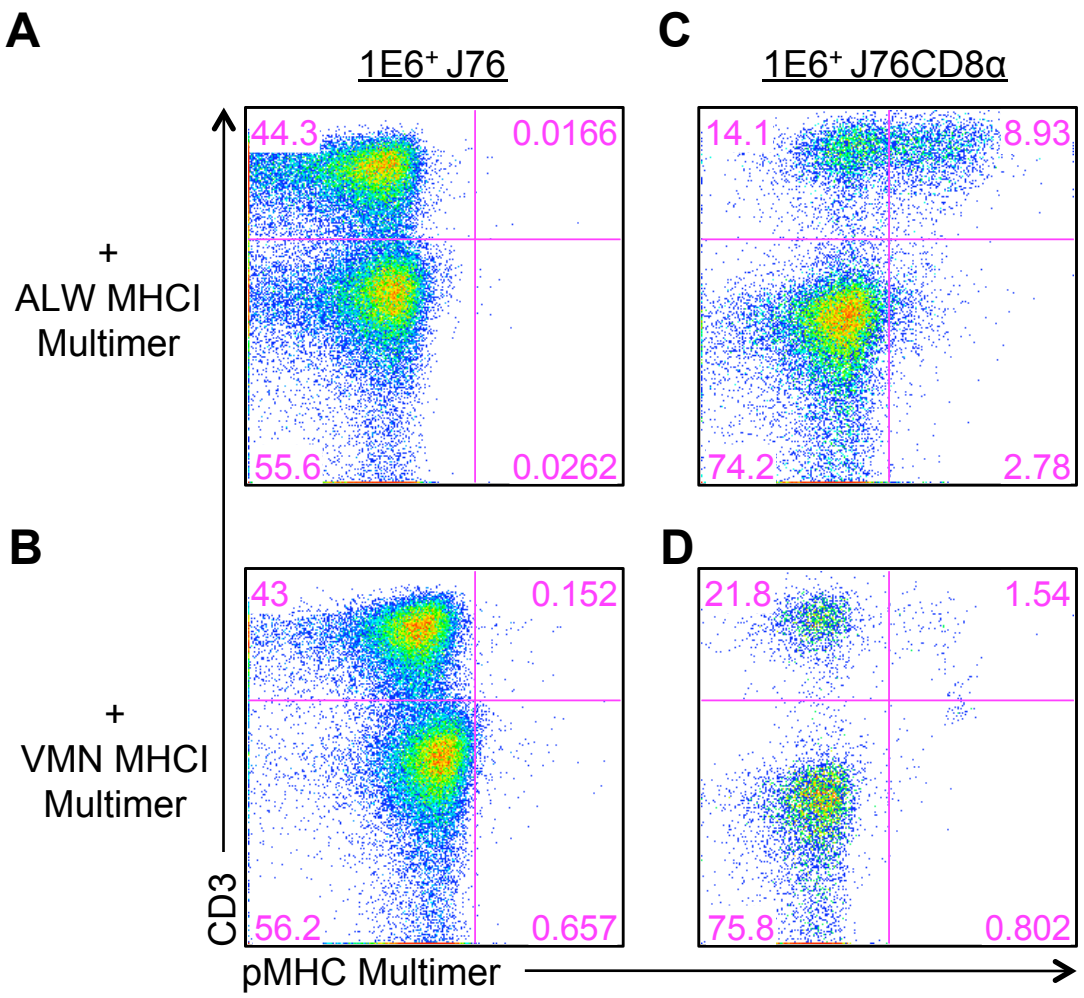

Figure 3-10 Staining of 1E6 TCR Transduced Jurkat Cells with an ALW MHCI Multimer is Dependent on CD8α expression. 1E6 transduced J76 cells and J76CD8α cells were stained with either (A) & (C) peptide specific ALW MHCI Multimer or (B) & (D) VMN MHCI Multimer as an irrelevant pMHC control

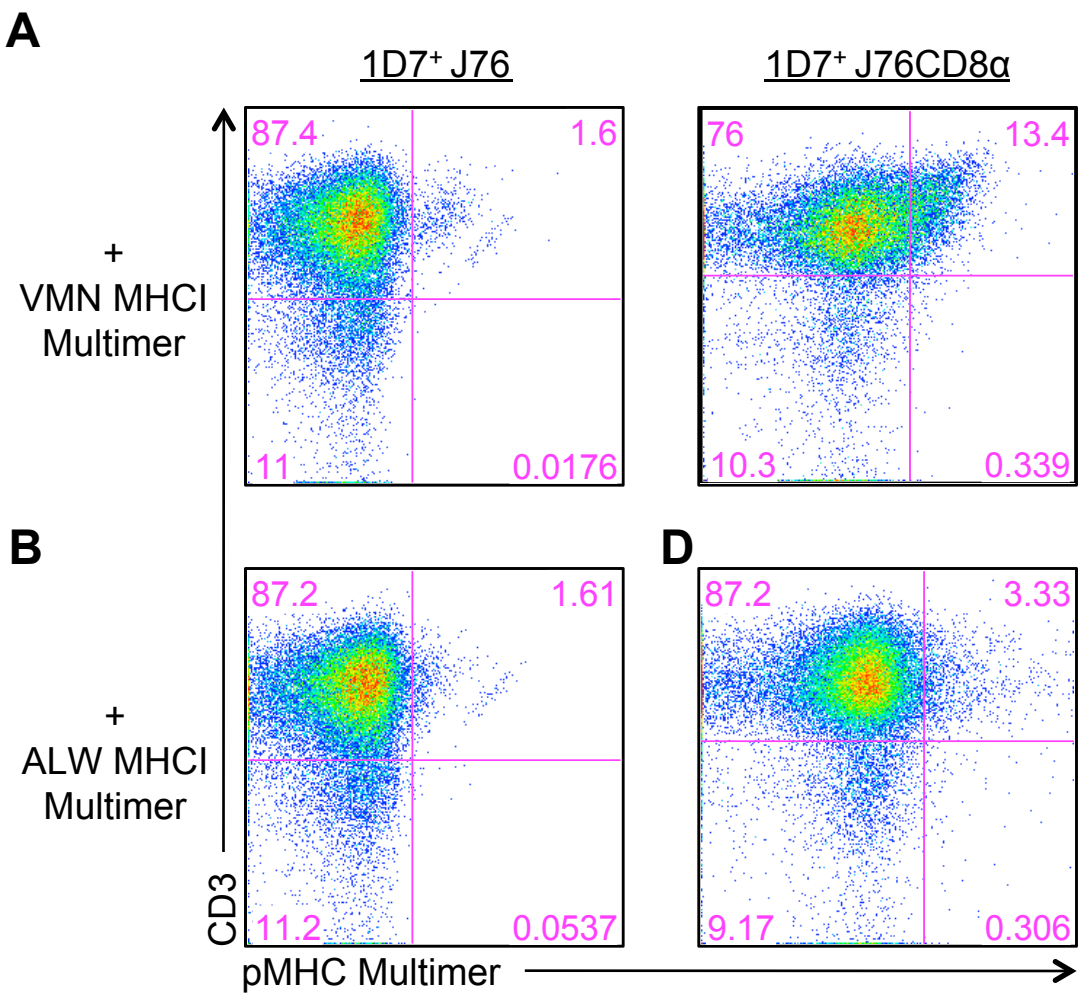

Figure 3-11 A VMN MHCI Multimer Identifies a Proportion of 1D7$^+$J76CD8α cells. 1D7 transduced J76 cells and J76CD8α cells were stained with either (A) & (C) peptide specific VMN MHCI Multimer or (B) & (D) ALW MHCI Multimer as an irrelevant pMHC control.

3.3.5. TCR Transduction of TCR$^-$ Jurkat Cell Lines Restores TCR Signalling Capabilities

The expression and function of a TCR$\alpha\beta$ on the surface of a T cell is dependent on its association with the CD3 signalling complex (CD3$\delta\varepsilon$, CD3$\gamma\varepsilon$ and CD3$\zeta\zeta$) (Brenner et al., 1985; Geisler, 1992). The three subunits that make up the CD3 complex consist of 10 ITAMs, which account for TCR$\alpha\beta$ engagement induced signal transduction within a T cell. TCR$\alpha\beta^-$ Jurkat cell lines used in this study fail to express CD3 complex on their cell surface highlighting the dual reliance of TCR for the CD3 complex and vice versa. Thus, when these cells are re-constituted with TCR$\alpha\beta$ genes, their CD3 expression is rescued (Figure 3-12 (A) and (B)). Similar to the hierarchy of TCR expression between different introduced TCRs, the 1D7 TCR had the lowest CD3 expression with an MFI of 4861 and 5369 in J76 and J76CD8α respectively (Figure 3-12 (C), (D)). The levels of CD3 expression on the 1D7$^+$ Jurkat cell lines is much reduced in comparison to both 868$^+$ (11318 and 18391) and 1E6$^+$ (10176 and 13349) Jurkat cell lines.

It has now been demonstrated that the introduced TCRs were both expressed via staining with specific vβ antibodies and possess the correct conformation of the TCR α and β chains as evidenced by MHCI multimer staining. These results, in conjunction with successful co-expression of the CD3 signalling machinery allows for the assessment of these TCRs to function i.e. to signal in response to the TCRs specific cognate peptide.

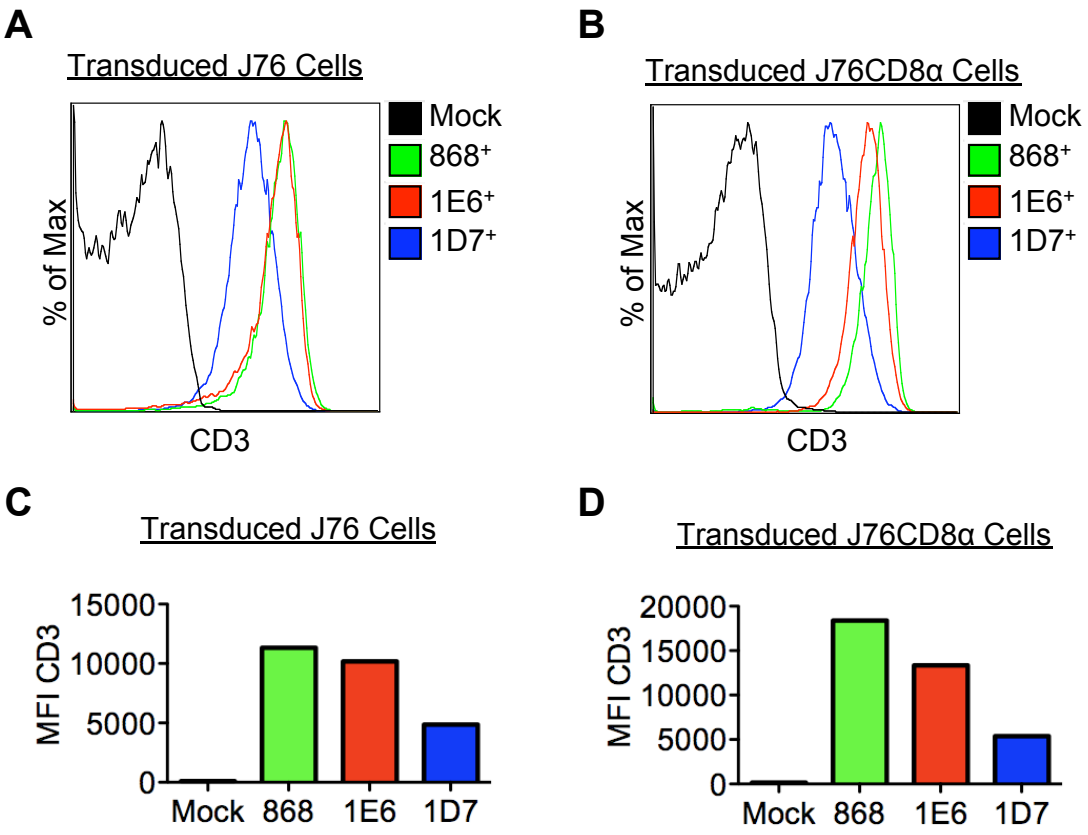

Figure 3-12 Transduced TCR$^-$ Jurkat cells up-regulate the CD3 complex upon TCR introduction. Jurkat cell lines were incubated with anti CD3 antibody to assess expression of the CD3 complex on transduced (A) J76 and (B) J76CD8α cells. The mean fluorescence intensity (MFI) of CD3 expression on (C) J76 and (D) J76CD8α cells was calculated. Data is representative of two individual staining occasions.

3.3.6. High Affinity MHC Class I Restricted TCR can be Activated by Cognate Peptide Irrespective of CD8α Expression

To investigate whether the TCR transduced Jurkat T cell lines could be activated by their cognate peptide, the up-regulation of the lymphoid activation marker, CD69, was assessed. The CD69 marker is known to be an early marker of T cell activation and its expression has been demonstrated by flow cytometry 4 hours post TCR engagement (Simms and Ellis, 1996). To determine the optimal time-point to assess CD69 up-regulation on the Jurkat cell lines, 868^{+}J76CD8α cells were activated by the SL9 peptide and CD69 up-regulation measured over time. The assay set up detailed in Figure 3-3 was used and replicate wells harvested 4,8, 16 and 20 hours post stimulation. The optimal time-point for CD69 expression was deemed to be 16 hours as at 20 hours expression had reduced (Figure 3-13). This time-point was then used for all subsequent activation assays. To test the 868 TCR was functional within the Jurkat cell lines, both the 868^{+} J76 and 868^{+}J76CD8α cell lines were stimulated with PBMC pulsed with, PBS, CytoStim or SL9. CytoStim is an antibody-based product, which promotes TCR activation by binding to TCRs and cross-linking them to MHC molecules. Thus, CytoStim can be used as a positive control to verify that the introduced TCRs are capable of signalling. From initial FACS staining, CD69 up-regulation can be seen in response to both CytoStim and the SL9 cognate peptide (Figure 3-14 (A)). The ability of the SL9 peptide to elicit CD69 up-regulation in the presence and absence of CD8α highlights that the 868 TCR can be classed as a CD8 independent TCR, a result that has been previously shown in primary cells and ties in with the pattern of pMHC multimer staining (Plesa et al., 2012). Interestingly the activation of the 868 TCR was consistently higher in the $CD8\alpha^{-}$ Jurkat cells compared to the $CD8\alpha^{+}$ Jurkat cells ($P < 0.0001$) (Figure 3-14 (B)).

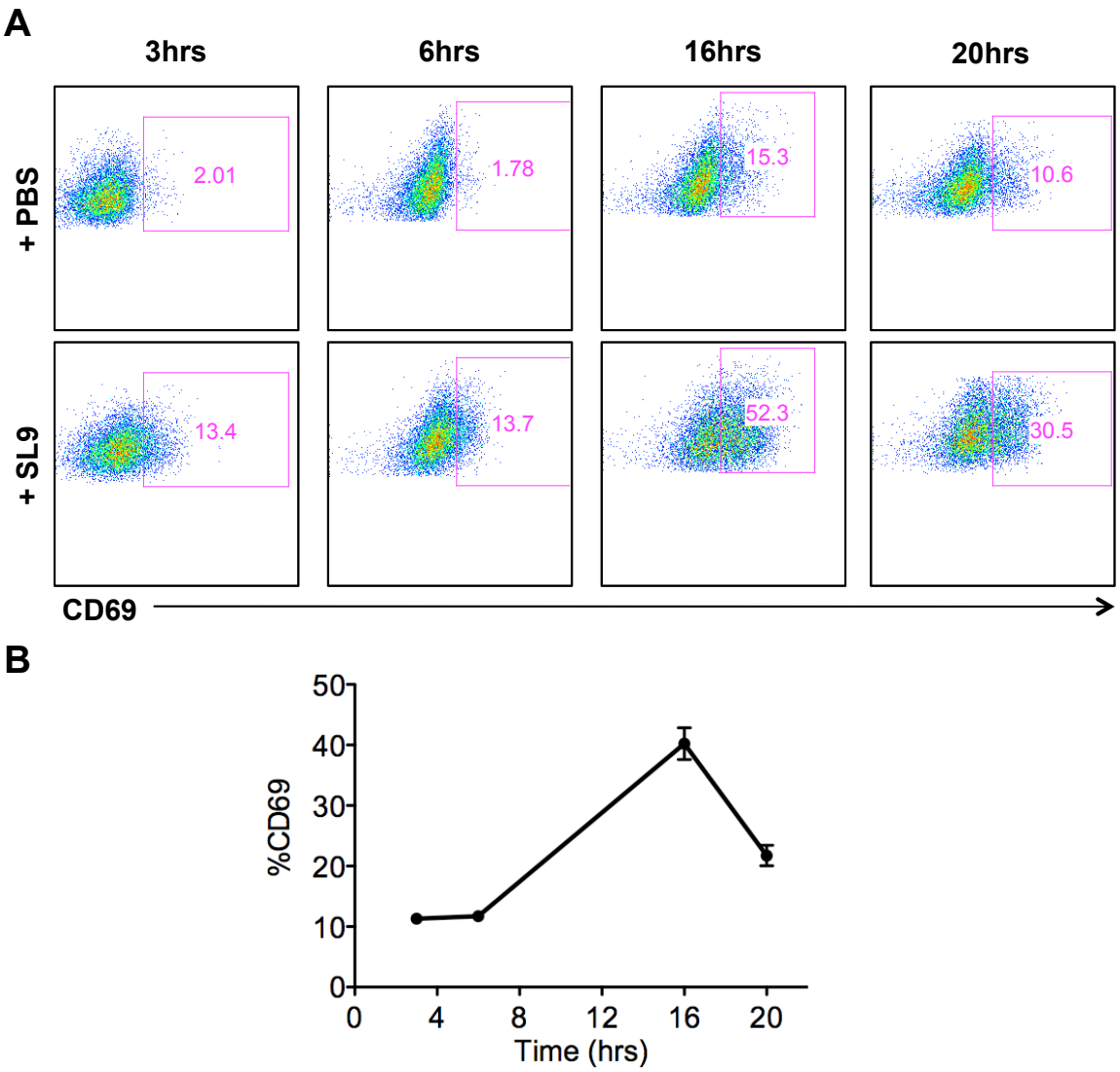

Figure 3-13 Time course for CD69 up-regulation following peptide specific activation of Jurkat cell lines. 868^{+}J76CD8α Jurkat cell lines were activated with Violet labelled PBMCs pulsed with cognate peptide or PBS as a negative control. (A) Representative FACS plots of CD69 expression of stimulated 868J76CD8α cells at indicated time points. Data is representative of three independent experiments and CD69 expression was normalised to the PBS stimulated control by subtraction of stimulation by PBS at each time point for (B) 868J76CD8α activation with SL9 peptide.

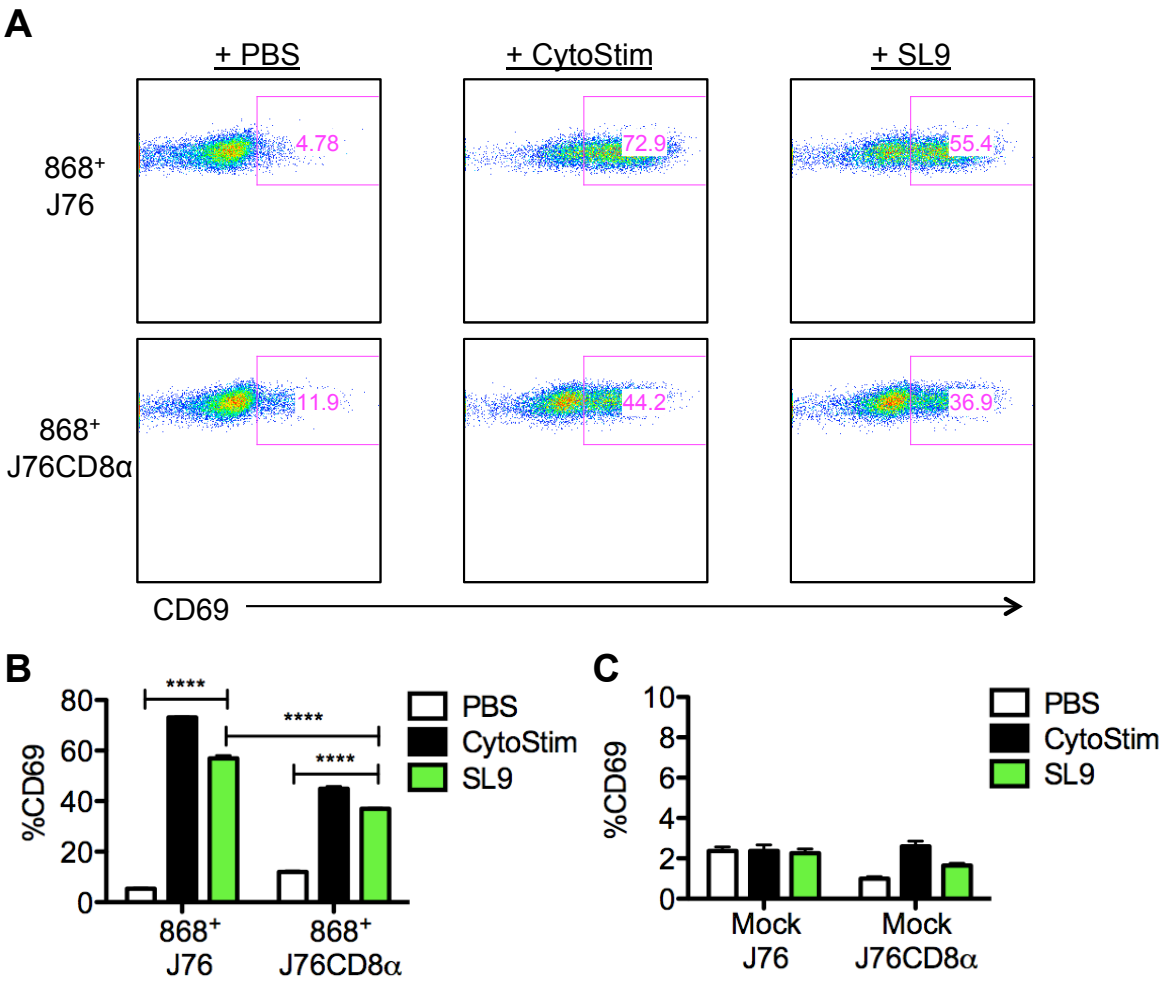

Figure 3-14 Activation of 868 Transduced Jurkat Cells. 868 transduced J76 and J76CD8α were incubated with violet labelled PBMCs pulsed with PBS, CytoStim and index peptide SL9. CD69 expression on Jurkat cells was measured by Flow Cytometry 16 hours post activation. (A) Representative FACS plots show activation of 868^+ J76 and 868^+ J76CD8α cells activated with PBS, CytoStim and SL9. (B) Data is representative of three independent experiments, and the mean of one experiment shown. (C) Mock transduced J76 and J76CD8α cells were stimulated as described and data represents two independent experiments. An unpaired Student's t test was used to calculate data significance. P Value = **** <0.0001.

3.3.7. Reduced Responsiveness of Autoreactive MHCI Restricted TCRs

The autoreactive 1E6 and 1D7 expressing Jurkat cells were similarly assessed for their ability to up-regulate CD69 in response to stimulation with cognate peptide. Both the $1E6^+$ J76 and $1E6^+$ J76CD8α were used in this assay and stimulated with PBS, CytoStim and the ALW peptide (Figure 3-15 (A)). Only the $1E6^+$J76CD8α cells, and not the $1E6^+$J76 cells, were able to respond to the ALW peptide, highlighting the dependence of this TCR on CD8α expression. This result was consistent over multiple experiments and in all cases the 1E6 TCR only responded to ALW peptide via CD69 up-regulation in the presence of the CD8α co-receptor (Figure 3-15 (B)). This response was TCR specific as neither Mock J76 or Mock J76CD8α cells responded to PBMCs presenting the ALW peptide (Figure 3-15 (C)). In contrast to this, the $1D7^+$ Jurkat cells were incapable of up-regulating CD69 in response to the VMN peptide, even in the presence of the CD8α co-receptor (Figure 3-16 (A-B). Both $1D7^+$ Jurkat cells could up-regulate CD69 in response to CytoStim however, showing that this TCR was functional. To compare the dose responsiveness of the three TCRs to their cognate peptide an 8-log fold dose titration was performed (Figure 3-17). A starting concentration of 100μM was chosen as a dose 10-fold higher than the concentrations previously used, to rule out any lack of response due to minimal peptide concentration. The response of both 868^+J76 and 868^+J76CD8α cells to the SL9 peptide were similar in magnitude, again highlighting that CD8α is not required for 868 TCR binding to MHCI and SL9 (Figure 3-17 (A)).

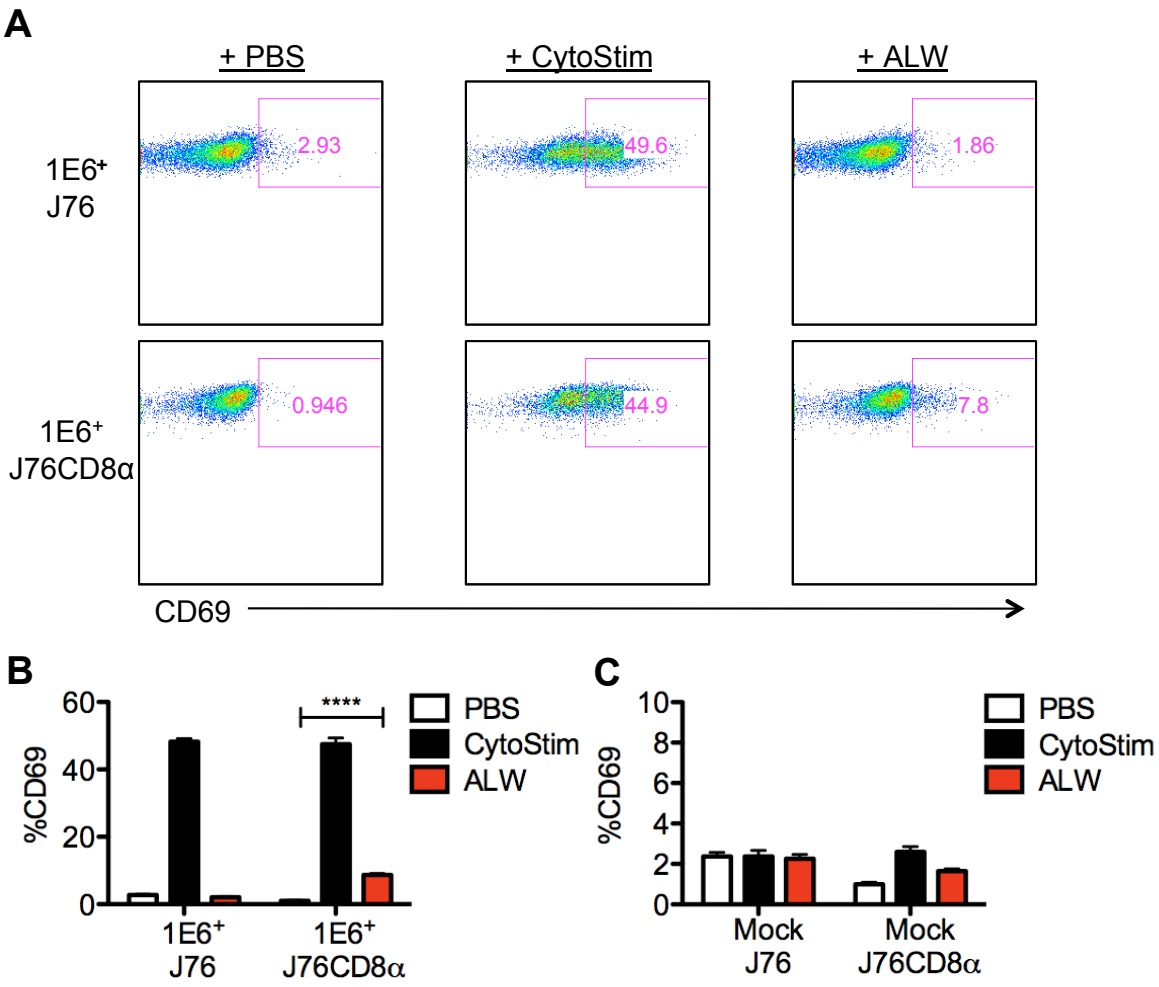

Figure 3-15 Activation of 1E6 Transduced Jurkat Cells. 1E6 transduced J76 and J76CD8α were incubated with violet labelled PBMCs pulsed with PBS, CytoStim as a positive control and index peptide ALW. CD69 expression on Jurkat cells was measured by Flow Cytometry 16 hours post activation. (A) Representative FACS plots show activation of $1E6^{+}$ J76 and $1E6^{+}$ J76CD8α cells activated with PBS, CytoStim and ALW. (B) Data is representative of three independent experiments, and the mean of one experiment shown. (C) Mock transduced J76 and J76CD8α cells were stimulated as described and data represents two independent experiments. An unpaired Student's t test was used to calculate data significance. P Value = **** <0.0001.

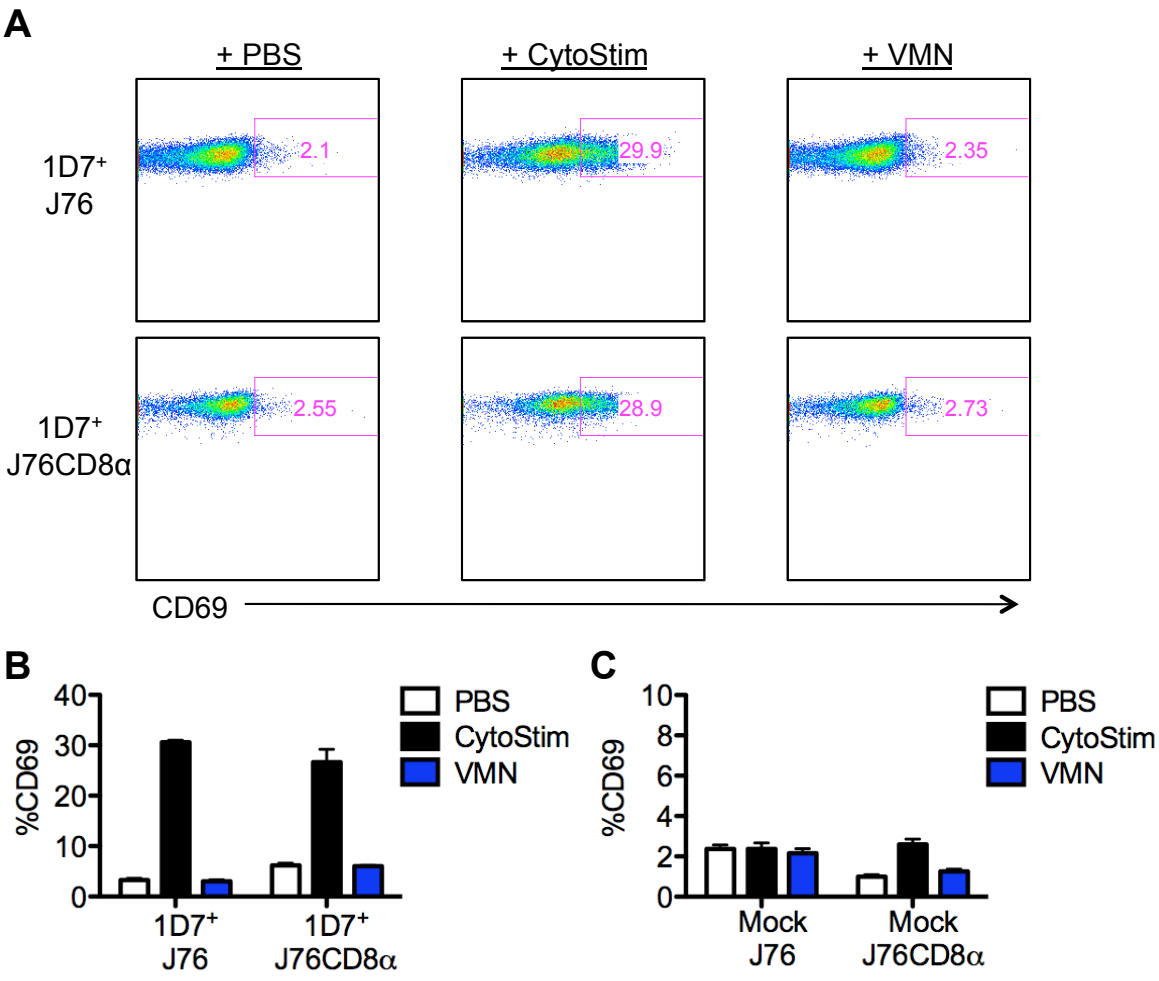

Figure 3-16 Activation of 1D7 Transduced Jurkat Cells. 1D7 transduced J76 and J76CD8α were incubated with violet labelled PBMCs pulsed with PBS, CytoStim as a positive control and index peptide VMN CD69 expression on Jurkat cells was measured by Flow Cytometry 16 hours post activation. (A) Representative FACS plots show activation of $1D7^+$ J76 and $1D7^+$ J76CD8α cells activated with PBS, CytoStim and VMN. (B) Data is representative of three independent experiments, and the mean of one experiment shown. (C) Mock transduced J76 and J76CD8α cells were also stimulated as described and data represents two independent experiments.

The response of the $1E6^+$J76CD8α cells in response to ALW peptide failed to reach maximum responsiveness even at the high dose of peptide used, suggesting a low affinity interaction between TCR and peptide (Figure 3-17 (B)).

Meanwhile, the high dose of peptide used failed to yield any response from the $1D7^+$ Jurkat cells (Figure 3-17 (C)).

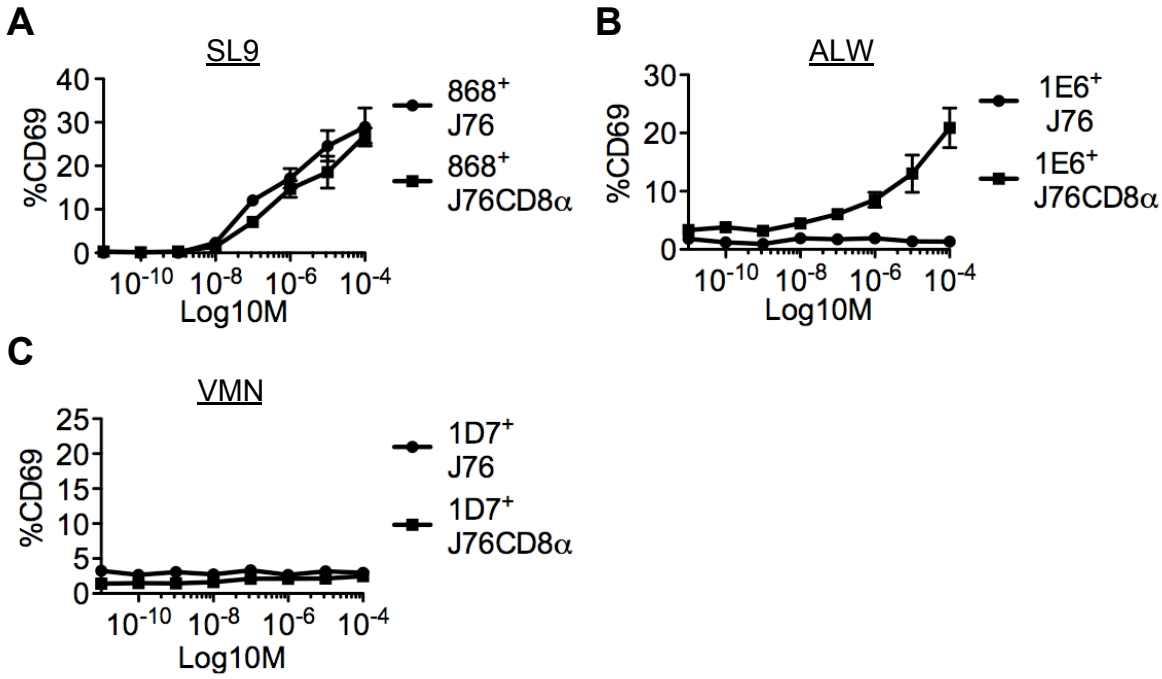

Figure 3-17 Dose Response Curves of TCR Transduced Jurkat cells to Cognate Peptide. TCR transduced J76 and J76CD8α cells were stimulated with 8 log fold dilutions of cognate peptide. (A) 868 transduced J76 and J76CD8α cells were stimulated with SL9 peptide, (B) 1E6 transduced cells with ALW and (C) 1D7 transduced cells with VMN peptide. Data is from two independent experiments.

3.3.8. Heteroclitic Peptide can be used to Activate the 1E6 TCR

Since its isolation, the 1E6 $CD8^+$ T cell clone has been used in a variety of studies aiming to characterise autoreactive $CD8^+$ T cells. One such study demonstrated that the 1E6 $CD8^+$ T cell clone was capable of recognising more than a million different peptides (Wooldridge et al., 2012). Moreover, by employing a decamer combinatorial peptide library *Wooldridge et al* identified a range of decamer peptides that were more potent at activating the 1E6 $CD8^+$ T cell clone. Two of these peptides, termed super agonist peptides, were chosen for this project and are detailed in Table 3-3. The RQF peptide was selected due

to its far superior potency at activating the 1E6 $CD8^+$ T cell clone compared to the ALW peptide, and the YQY peptide was selected as a medium activator of this TCR. These three peptides were then used to stimulate $1E6^+$ J76 and $1E6^+$ J76CD8α cells (Figure 3-18 (A)). Most interestingly, the RQF peptide could stimulate both the $1E6^+$J76 and $1E6^+$J76CD8α cells to up-regulate CD69, whereas the YQY and ALW peptide were only capable of activating the $1E6^+$J76CD8α cells (Figure 3-18 (B)). To further investigate the potency of each peptide for activating the 1E6 TCR, $1E6^+$J76CD8α cells were stimulated with log dose titrations of the three peptides. These results show that the RQF peptide could activate the $1E6^+$J76CD8α cells at much lower peptide concentrations (10nM/ml) than either the ALW or YQY peptide (1-10μM/ml) (Figure 3-18 (C)). Furthermore, the maximum response achieved by the $1E6^+$J76CD8α cells when stimulated by either ALW or YQY peptide, ~20%, could be reached with four logs lower concentration of RQF peptide. Additionally, the maximum response reached by the $1E6^+$J76CD8α cells when stimulated with the RQF peptide was much higher (Mean 65 ± 5.7% SD) compared to stimulation with the maximum concentration of ALW (Mean 20.9 ± 5.3% SD). These results therefore suggest that the interaction between the 1E6 TCR and the RQF peptide is of such a high affinity that it does not require the CD8α co-receptor. Surprisingly, the YQY peptide, that was proffered as a medium activator of the 1E6 TCR, was no better at activating this TCR than the cognate ALW peptide, and in fact the maximal response to this peptide was lower than to the ALW peptide (Figure 3-18 (C)).

Table 3-3 A list of the super agonist peptides used in their study and their relative functional sensitivity calculated as pEC50(agonist) - pEC50(index peptide) (Wooldridge et al., 2012)

Peptide	Sequence	Relative Functional Sensitivity
ALW	ALWGPDAAA	0
YQY	YQYGPDFINA	1.5
RQF	RQFGPDFPTI	3

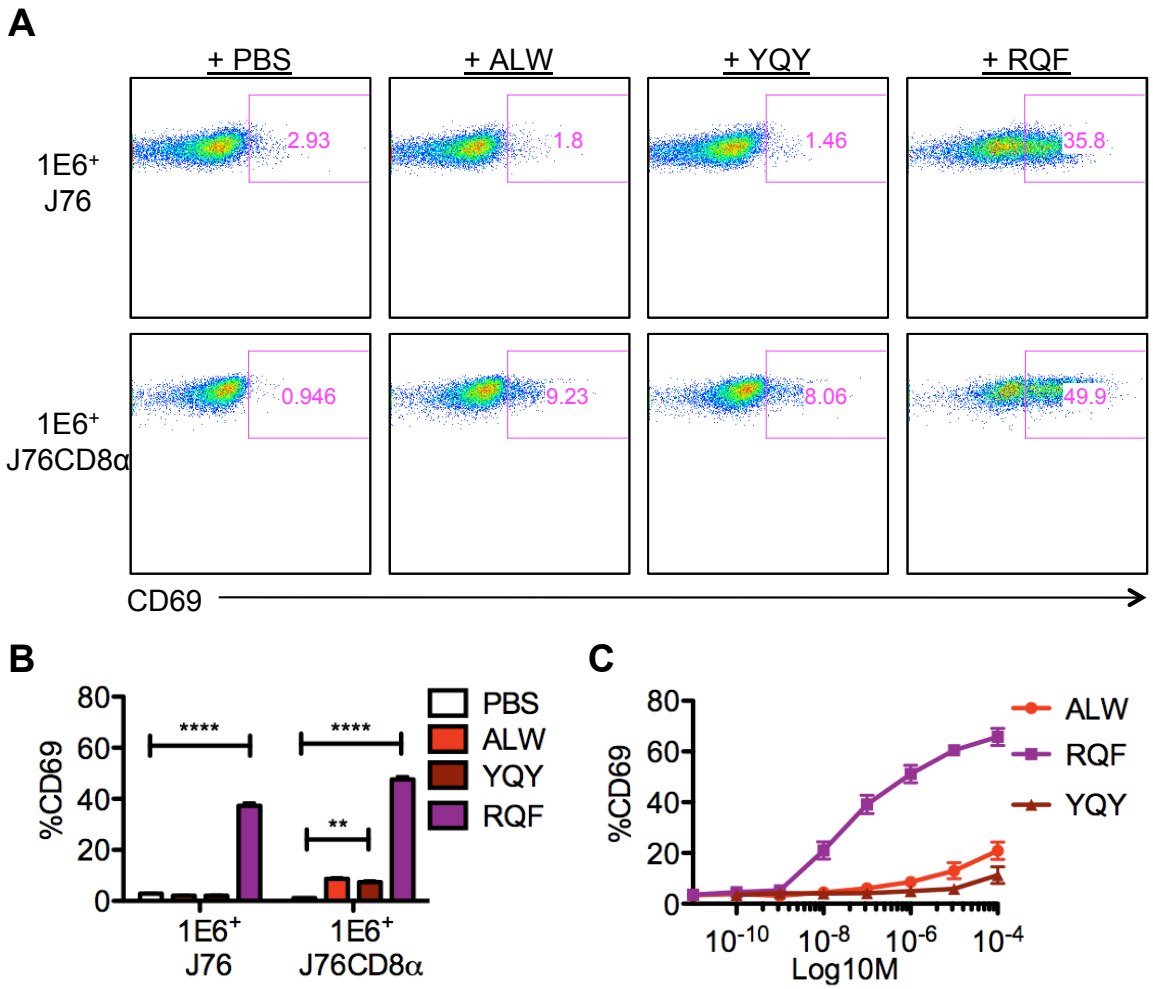

Figure 3-18 Activation of 1E6 Transduced Jurkat Cells with Super Agonist Peptides. The 1E6 clone from which the 1E6 TCR was isolated has previously been shown to have a greater functional sensitivity to super agonist peptides than to the WT peptide (Wooldridge et al., 2012). (A) 1E6 transduced Jurkat cells were activated as previously with the WT ALW peptide and super agonist YQY and RQF peptides. (B) Data is representative of three independent experiments. (C) To measure the functionally sensitivity of the 1E6 TCR transduced J76CD8α these cells were stimulated with eight log fold dilutions of ALW RQF and YQY. Data is representative of three independent experiments. An unpaired Student's t test was used to calculate data significance. P Value = ** < 0.01, **** <0.0001.

3.3.9. The Activation of Autoreactive TCRs can not be Rescued by LV Co-expression of CD8α

The observation that 1E6$^+$J76CD8α cells could respond to the ALW peptide led us to create a LV vector whereby CD8α could be co-expressed with the 1E6 TCR in any cell type of choice. This LV vector, designated 1E6.CD8α consisted of the 1E6 TCRα and TCRβ chains linked to a CD8α fragment (Figure 3-19 (A)). To generate this vector, the pELNS.1E6.RatCD2 vector was first digested with NsiI and SalI restriction enzymes to remove the RatCD2 gene from this plasmid (Figure 3-19 (B)). A CD8α gene with 5' NsiI and 3' SalI restriction sites was a kind gift from Linda Wooldridge (University of Bristol). The prepared vector and CD8α gene fragment were ligated and the ligation products were transformed into competent cells, from which the plasmid DNA was prepared. The prepared DNA was digested with NsiI and SalI restriction enzymes to resolve the CD8α fragment (Figure 3-19 (C)). The pELNS.1E6.RatCD2 vector was also digested as a control. The Rat CD2 gene (705bp) and CD8α gene (712bp) were too close in size to determine by agarose gel if the CD8α gene had successfully replaced the Rat CD2 gene (Figure 3-19 (C)). 293Ts were transfected with the prepared DNA and transfected cells were stained with Rat CD2 and CD8α to confirm expression of CD8α over Rat CD2 (Figure 3-19 (D)). A positive plasmid preparation was then selected as the pELNS.1E6.CD8α vector. To test if the LV co-expression of CD8α could rescue the response of the 1E6 TCR, J76 cells were transduced with the 1E6.CD8α LV. These cells were then stimulated with either PBS or ALW to assess for CD69 up-regulation. 1E6$^+$J76 and 1E6$^+$J76CD8α cells served as a negative and positive control respectively. After the cells had been activated for 16 hours, they were stained with antibodies against CD8α and CD69 (Figure 3-20 (A)). This allowed for analysis of CD69 up-regulation in 1E6$^+$CD8α$^+$ cells only. In three individual experiments, no CD69 up-regulation was seen in cells co-expressing 1E6 and CD8α, from the LV vector, in response to ALW peptide (Figure 3-20 (B)). However, as before, CD69 up-regulation was seen in the 1E6$^+$J76CD8α cells. Upon closer analysis of CD8 expression between these two

cell lines, a difference in CD8 expression became clear. Overlays of histograms of CD8 MFI expression on 1E6^{+}J76CD8α cells and 1E6.CD8α^{+}J76 cells revealed that the CD8 expression from the 1E6.CD8α LV vector was markedly reduced compared to the J76CD8α cell line (Figure 3-20 (C)).

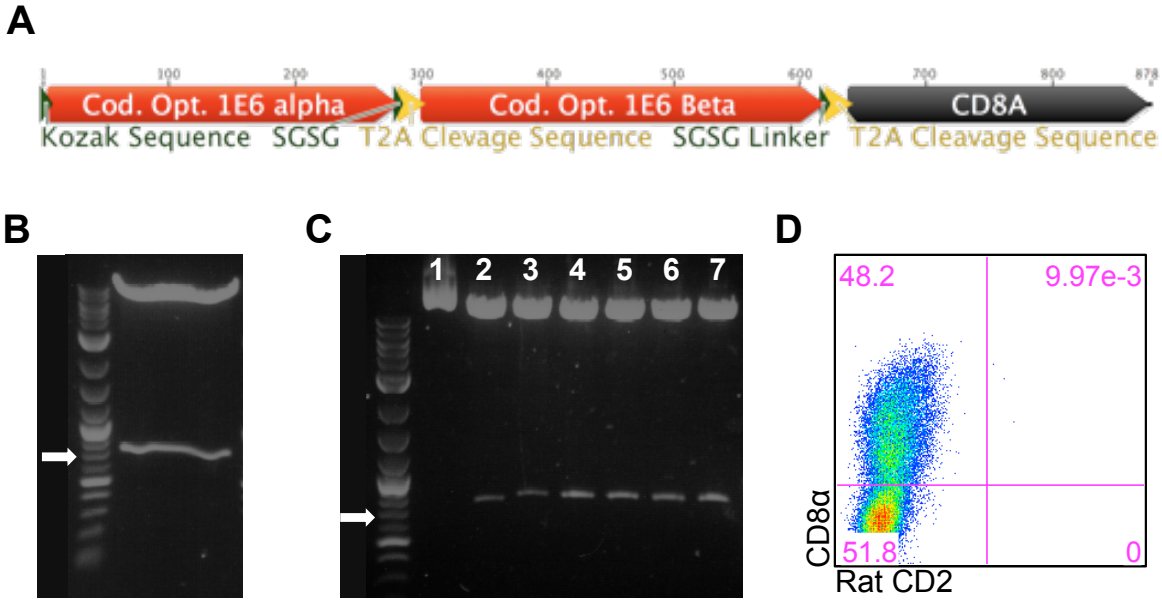

Figure 3-19 Generation of 1E6.CD8α LV Vector. (A) A CD8α gene fragment was cloned into the 1E6 pELNS LV vector and linked to the 1E6 TCRβ chain by a T2A cleavage site. The resulting LV vector was designated 1E6.CD8α (B) To generate the vector the pELNS.1E6.RatCD2 vector was digested with NsiI and SalI to remove the 705bp Rat CD2 fragment as indicated by the white arrow (C) the digested pELNS.1E6 vector and CD8α fragment with 5' NsiI and 3'SalI restriction sites were ligated, transformed into competent cells and the plasmid DNA prepared. Gel shows: Lane 1 - pELNS.1E6.RatCD2 vector uncut, Lane 2 - pELNS.1E6.RatCD2 digested with NsiI and SalI and Lane 3-7 show ligation products digested with NsiI and SalI. White arrow shows the 700bp marker. (D) A representative FACS plot of 293T cells transfected with prepared DNA from the pELNS.1E6 and CD8α ligation, stained with CD8α and Rat CD2 antibodies. The left hand lane of both gels were loaded with a 2-log DNA ladder.

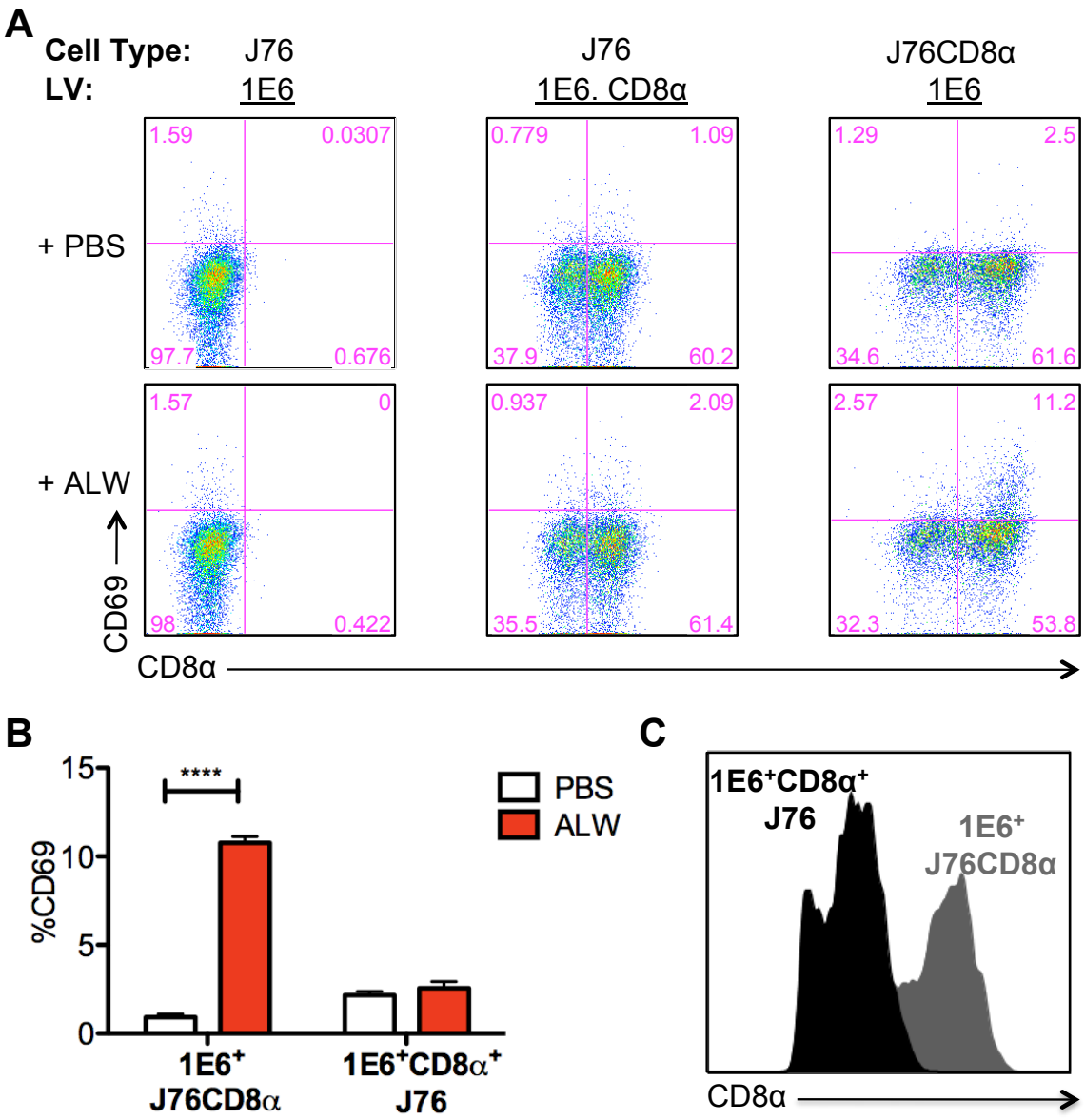

Figure 3-20 Activation of 1E6.CD8α transduced J76 cells. 1E6.CD8α transduced J76 cells were activated alongside $1E6^{+}$J76 and $1E6^{+}$J76CD8α cells with violet labelled PBMCs pulsed with either PBS as a negative control of the 1E6 TCR cognate peptide ALW. (A) Representative FACS plots show activated Jurkat cells stained with antibodies against CD69 and CD8α. (B) CD69 up-regulation was analysed within the $CD8α^{+}$ population of both $1E6^{+}$J76CD8α and $1E6^{+}CD8α^{+}$J76 cells. Data is representative of three independent experiments. P Value = **** < 0.0001 (C) CD8α expression was assessed on 1E6.CD8αJ76 cells (Black histogram) and $1E6^{+}$J76CD8α cells (grey histogram).

3.4. Discussion

The experiments in this chapter were designed to test the three MHCI TCRs that will be used throughout this study. The use of TCR$\alpha\beta^-$ Jurkat cells as model cell lines was useful to verify TCR constructs as any expression differences caused by competition with the endogenous TCR are removed. Using these cells, each TCR construct was examined for the ability to be fully expressed and to re-direct the Ag specificity of the model cell lines. These experiments revealed that the TCRα and TCRβ chains of each TCR could successfully pair and fold to form a complete cell surface TCR, and additionally they could successfully pair with the CD3 signalling complex. Interestingly, despite a lack of competition for TCR expression on the cell surface of the Jurkat cells there was a stark difference in TCR expression between the three TCRs tested. Expectedly, this low expression of TCR$\alpha\beta$ correlated with a low expression of the CD3 complex.

For a TCR to be successfully expressed on the surface of a T cell two crucial events must take place. Firstly the TCRα and TCRβ chains (which constitute the TCR) must successfully pair with one another and secondly this TCR must form non covalent bonds with all the subunits that create the CD3 complex. Only after these two processes take place can the optimal cell surface expression of the TCR-CD3 complex be observed (Ohashi et al., 1985; Saito et al., 1987). The model J76 cell lines used in the project are deficient in both TCRα and TCRβ genes, thus the introduced TCRs were under no competition for coupling to CD3. Therefore, the observation that the two autoreactive TCRs, 1E6 and particularly 1D7, were expressed at much lower levels than the pathogen specific 868 TCR could be due to an inefficient pairing of the TCRα and β genes of each TCR. It has been previously shown that expression of introduced TCRs in primary T cells is controlled intrinsically by the qualities of the introduced TCR (Heemskerk et al., 2007). Thus the hypothesis that the reduced expression of the two autoreactive TCRs is due to a reduced capacity of the TCRα and TCRβ chains of each TCR to pair is not unrealistic. A potential experiment to test this hypothesis could be to perform intracellular staining for the specific vβ of each TCR transduced Jurkat

cell line to determine whether the autoreactive TCRs' ability to traffic to the cell surface is compromised. However, it is known that unassembled TCR-CD3 complexes are targeted for degradation within the endoplasmic reticulum only a few hours post synthesis (Bonifacino et al., 1989), thus potentially skewing any results using intracellular staining.

After determining that the TCRs could be expressed on the cell surface of the Jurkat cell lines, it was examined whether the correct TCR conformation was displayed on the cells surface. To do this, pMHCI multimers were used to confirm the TCRs could bind to cognate pMHCI and PBMCs pulsed with peptide were used to demonstrate the signalling ability of TCR transduced cells in response to cognate peptide. These further experiments revealed a clear difference in the ability of the autoreactive TCRs to respond to cognate pMHC in comparison to the pathogen specific 868 TCR. The 868 TCR behaved equally in J76 and J76CD8α cells, which would be expected based on results seen by others in primary human T cell populations (Plesa et al., 2012). An SL9-MHCI multimer could positively stain both 868^{+}J76 and 868^{+}J76CD8α cell lines, demonstrating that this TCR was in the correct conformation on the surface of the Jurkat cells. Furthermore, both 868^{+} Jurkat cell lines could robustly and reproducibly up-regulate CD69 in response to PBMCs pulsed with SL9 peptide. These results demonstrated that the 868 TCR could re-direct the Ag specificity of Jurkat cell lines irrespective of CD8α co-receptor expression. The interaction between the 868 TCR and SL9-MHCI could therefore be classed as CD8 independent. Interestingly, the presence of CD8α in conjunction with the SL9 TCR increased non-specific activation- a result consistently observed when 868^{+} J76CD8α cells were incubated with PBMCs pulsed with PBS and when these cells were stained with an irrelevant pMHC multimer. The co-expression of CD8 and high affinity TCRs has previously been shown to decrease the Ag specificity of a TCR by increasing the length of interaction between TCR and pMHCI (Zhao et al., 2007). This phenomenon could also explain why the functional avidity of the 868 TCR,

as measured by SL9 dose titration, was comparable between 868^+ J76 and 868^+ J76CD8α cells.

Cells transduced with the 1E6 TCR were found to be completely reliant on the presence of CD8α for the recognition of the WT ALW peptide. The $1E6^+$ J76CD8α showed a reproducible, yet modest response via CD69 up-regulation when presented with ALW peptide. Even when PBMCs were pulsed with the highest dose of peptide (100μM), the $1E6^+$J76CD8α did not reach maximal response to the WT peptide. These results mirror the data seen when these cells are stained with the ALW pMHCi multimer, with only a proportion of these cells capable of responding. This dependence of 1E6 on CD8α for functional response to ALW is unsurprising given its naturally low affinity for WT peptide (Bulek et al., 2012). It is worthwhile to mention that the function of CD8$\alpha\alpha$ homodimer as a co-receptor is controversial (Cheroutre and Lambolez, 2008) and there is evidence of CD8$\alpha\alpha$ acting as a suppressor of TCR activation in gut intra-epithelial T cells (Hayday et al., 2001). Studies using murine CD8β^- and CD8β^{wt} mice have shown that upon retroviral TCR transduction of CD8 dependent TCRs, these TCRs can only function in CD8β^{wt} T cells. However, the ability of CD8$\alpha\alpha$ to interact with HLA-A2*01 molecules and stabilise the TCR:pMHCI interaction has been well demonstrated and it is this function that may be integral for allowing a functional response to ALW from the $1E6^+$J7CD8α cells (Gao et al., 1997). The ability of the 1E6 TCR to recognise peptides could also be rescued using previously identified peptides that were capable of activating the 1E6 $CD8^+$ T cell clone to a much greater degree (Wooldridge et al., 2012). Most interestingly, the RQF peptide that was found to activate the 1E6 $CD8^+$ T cell clone by >100 fold compared to WT peptide, was able to specifically activate both the $1E6^+$J76CD8α and $1E6^+$J76 Jurkat cell lines. Thus, the interaction between the 1E6 TCR and RQF-MHCI is of such a high affinity it bypasses the requirement for CD8 co-receptor. The second peptide chosen, YQY, was chosen due to its intermediate functional sensitivity for the 1E6 TCR of >10 fold compared to ALW. Surprisingly the results seen with the YQY peptide were comparable to those seen with the ALW peptide, in both the CD8α dependency and its functional avidity for 1E6 TCR. In fact, at the same

concentrations the ALW peptide could elicit a slightly greater response from the $1E6^{+}$J76CD8α cells than the YQY peptide.

The second autoreactive TCR, the $GAD65_{114\text{-}122}$ VMN specific 1D7 TCR, proved more of an anomaly than the 1E6 TCR. A modest percentage of these cells could be specifically identified with the VMN pMHCI multimer compared to staining with the irrelevant ALW MHCI multimer, however this positive staining was not as distinct as other positive TCR pMHC multimer stains. Despite this, cognate peptide pulsed PBMCs were unable to elicit a response from $1D7^{+}$J76 cells or $1D7^{+}$ J76CD8α cells, despite very high concentration used in the peptide dose titration studies. However, it is clear that this is a functional TCR capable of signalling as it exhibited a robust response when stimulated with CytoStim as a positive control. It is plausible that the observed poor expression of the 1D7 TCR and CD3 complex could be the cause of this result. T cell activation via the TCR is the result of multiple TCR triggering events that amplifies the TCR signalling cascade (Valitutti et al., 1995) and potentially the lack of 1D7 TCR expression could results in minimal TCR triggering that does not exceed the threshold required for TCR activation. Furthermore, the minimal positive staining with the VMN tetramer reveals an extremely low affinity interaction between the 1D7 TCR and VMN tetramer, which appears to be even lower that the 1E6 TCR affinity for ALW tetramer.

In this study the autoreactive MHCI multimers used were MHCI tetramers. MHCI tetramers are composed of four biotinylated pMHCI monomers that are assembled into a complex by streptavidin labelled fluorochromes. Since the discovery of the MHCI tetramer, other MHCI multimers have come onto the market such as pentamers, octamers and more recently dextramers (Batard et al., 2006). Dextramers consist of a dextran polymer backbone that carries a number of fluorochrome labelled pMHC molecules. The increased abundance of pMHC molecule ensures the dextramer has a higher avidity for binding to Ag specific TCRs. Recently, MHCI dextramers have proven to be superior to MHCI

tetramers at binding to Ag specific T cells that have a low affinity TCR pMHCI interaction (Dolton et al., 2014). In fact, this study used a 1E6 TCR^+ clone to identify this and showed that the ALW MHCI dextramer could bind 17x more efficiently to the 1E6 TCR than the ALW MHCI tetramer. However, in a single assay no difference was seen in the staining of $1E6^+$ Jurkat cells with ALW MHCI tetramers or ALW MHCI dextramers (data not shown). During this current study, no VMN MHCI dextramers were available to test their staining of the $1D7^+$ Jurkat cell lines although this would be a useful test should they become available. A second tool that is used to improve MHCI multimer binding is the use of protein kinase inhibitor Dastanib, which can prevent TCR down-regulation, thereby improving pMHCI multimer binding (Lissina et al., 2009). All MHCI multimer staining in this section however was carried out after pre-incubation of the cells with PKI. Moreover, a side-by-side comparison of $1D7^+$ Jurkat cells that were pre incubated +/- PKI and then stained with either VMN MHCI tetramer or a pan TCR$\alpha\beta$ antibody revealed no difference in the MFI of either reagent (data not shown). This suggests that PKI does not affect the expression of the introduced TCRs in Jurkat cell lines. Another interesting observation that has come from previous studies using MHCI multimers identified that the affinity threshold required for pMHCI tetramer binding was higher than that required for TCR activation (Laugel et al., 2007). This is in contrast to the results seen in this current study. Indeed, a higher percentage of $1E6^+$J76CD8α cells could stain with ALW MHCI tetramer (40%) over those that could be activated by the ALW peptide to up-regulate CD69 (20%). Additionally, $1D7^+$J76CD8α cells could bind VMN MHCI tetramer but could not up-regulate CD69 in response to this peptide. Although, this could be due to the use of the CD8α co-receptor, these results potentially demonstrate a difference between model Jurkat cell lines and autoreactive $CD8^+$ T cells in that autoreactive CD8 T cells may have intrinsic properties that make them more susceptible to peptide activation.

The observation that the $1E6^+$J76CD8α cells could respond to the ALW peptide pulsed PBMCs led us to try to recapitulate this result using LV co-expression of

CD8α. To this end the CD8α gene was inserted in the place of Rat CD2 in the pELNS.1E6 LV vector. Although using this vector the 1E6 TCR and CD8α could be co-expressed in J76 Jurkat cells, the expression of CD8α was not sufficient to allow signalling in response to the ALW peptide. Upon analysis of the CD8α expression, this gene was found to be markedly reduced in the LV transduced cells compared to the J76CD8α cell line. Therefore, the level of CD8α expression in the transduced cells may not be adequate to facilitate the stability of pMHCI and 1E6 TCR. On closer inspection of FACS plots whereby the $1E6^+$ J76CD8α cells had been activated by ALW, only the highest CD8α expressing cells responded to the peptide, a level of expression the transduced gene failed to reach. The reduced expression of CD8α after LV co-expression may be because it was the third gene to be expressed in the multi-cistronic pELNS.1E6.CD8α vector. Others have shown that when using tetra-cistronic vectors all four genes can be equally co-expressed on a transduced cell (Fisicaro et al., 2011). However, another study identified that the expression of downstream genes can be affected if the upstream genes are not efficiently expressed (Hurh et al., 2013). In fact, retrospective review of Rat CD2 expression in the TCR transduced cells reveals a much lower expression of this marker compared to mock transduced cells. This may suggest that the either the TCRα or TCRβ genes are not efficient at being expressed which subsequently affects the expression of the marker gene.

This section set out to thoroughly test the three MHC Class I TCR constructs at their ability to be expressed in model Jurkat cell lines and subsequently re-direct the Ag specificity of these cells. Through these data, it has been shown that all TCRs are functional, however the results show a consistent pattern in which islet Ag specific TCRs behave differently to a pathogen specific TCR. Despite these observed differences, the results in Jurkat cell lines provide a basis for the commencement of LV MHC class I TCR transduction of human $CD4^+$ T_{REG} cells.

4. LV TCR Transduction and Re-direction of the Antigen Specificity of Human Primary T Cell Populations.

4.1. Introduction

The second section of this study will investigate the transduction of primary human T cell populations with the previously described MHCI TCRs. Both the expression of these TCRs will be examined and their ability to redirect the Ag specific responses of human T cells, including conferring suppressive capabilities to human $CD4^+$ T_{REG} cells. The positive control 868 TCR was identified as a CD8 co-receptor independent TCR and thus is an invaluable tool for the progression of this thesis. However, it was seen in Jurkat cells, that the two autoreactive TCRs had different properties compared to the 868 TCR: their expression in TCR^- Jurkat cells was lower and the 1E6 TCRs response relied on the CD8α co-receptor. Furthermore, the GAD specific 1D7 autoreactive TCR failed to signal in response to cognate peptide even in the presence of CD8α, despite some indication of correct expression as evidenced by pMHCI multimers.

Although these data may suggest that MHCI restricted autoreactive TCRs may be unsuitable for the re-direction of $CD4^+$ T cells, a recent study by *Plesa et al* identified that $CD4^+$ T_{REG} cells may have altered TCR signalling requirements compared to $CD4^+$ T_{EFF} cells. For part of this study, the authors used LV gene transfer to transfer two MHCI restricted TCRs into human $CD4^+$ T_{REG} cells (Plesa et al., 2012). One of these TCRs, as previously mentioned was the 868 TCR and the second was a TCR specific for the tumour Ag NY-ESO-$1_{157-164}$. The NY-ESO-1 specific TCR has previously been shown to have a relatively low affinity for its cognate peptide (32μM compared to 868 TCR affinity for SL9 of 85nM) (Zhao et al., 2007). To investigate whether this low affinity TCR-pMHCI interaction could successfully re-direct the Ag specificity of $CD4^+$ T_{EFF} cells, the authors transfected

$CD4^+$ T_{EFF} cells with a LV vector encoding the NY-ESO-1 specific TCR. Interestingly, despite high levels of TCR expression (assessed by specific TCRvβ up-regulation) the NY-ESO-1-TCR transfected $CD4^+$ T_{EFF} cells were undetectable by staining with an NY-ESO-1 pMHCI multimer. Furthermore, these cells were unable to mount an effector response, as measured by the secretion of IFN-γ and IL-2 when incubated with K562-A2 presenting the NY-ESO-1 peptide. However, under the same conditions, NY-ESO-1 TCR transfected $CD8^+$ T cells mounted an effective effector response, suggesting the interaction of this TCR with its cognate peptide was dependent on the CD8 co-receptor. Most surprisingly, upon transfection of the NY-ESO-1 specific TCR into isolated $CD4^+$ T_{REG} cells, this TCR was capable of re-directing the Ag specific suppressive capabilities of these cells. This evidence led the authors to hypothesise that $CD4^+$ T_{REG} cells may have different TCR-pMHCI affinity requirements to a $CD4^+$ T_{EFF} cell.

This hypothesis was further supported by evidence suggesting that compared to $CD4^+$ T_{EFF} cells, $CD4^+$ T_{REG} cells have a lower threshold for TCR activation due to the occurrence of TCR signalling in the absence of the CD4 co-receptor. For example, mice that were transfused with a CD4 blocking antibody developed a specific accumulation of $CD4^+$ T_{REG} cells (Oliveira et al., 2011). It was further suggested that TCR signalling can occur in mature $CD4^+$ T_{REG} cells in the absence of CD4 recruitment of Lck, an important molecule in the TCR signalling cascade (Artyomov et al., 2010). These data therefore suggest that low levels of TCR triggering and signalling are sufficient to fully activate the suppressive function of T_{REG} cells. In theory, if an MHCII restricted TCR can signal in the absence of CD4 recruited Lck in a T_{REG} cell, it is possible that an MHCI restricted TCR may also signal in a co-receptor independent manner in the setting of a T_{REG} cell. Therefore, an MHCI restricted TCR that may require CD8 recruitment of Lck to aid signalling in a $CD8^+$ T cell, may not have the same requirements in a T_{REG} cell (Figure 4-1).

These results have therefore formed the working hypothesis for this study which is that an autoreactive MHCI restricted TCR can transfer Ag specificity to a $CD4^+$

T_{REG} cell, despite its reliance on CD8α in model Jurkat cell lines – a hypothesis which will be thoroughly tested in this section of this thesis. This will be tested not only by assessing the functional response of TCR transduced $CD4^+$ T_{REG} cells but also by testing the ability of these TCRs to signal in $CD4^+$ T_{EFF} and $CD8^+$ T cells

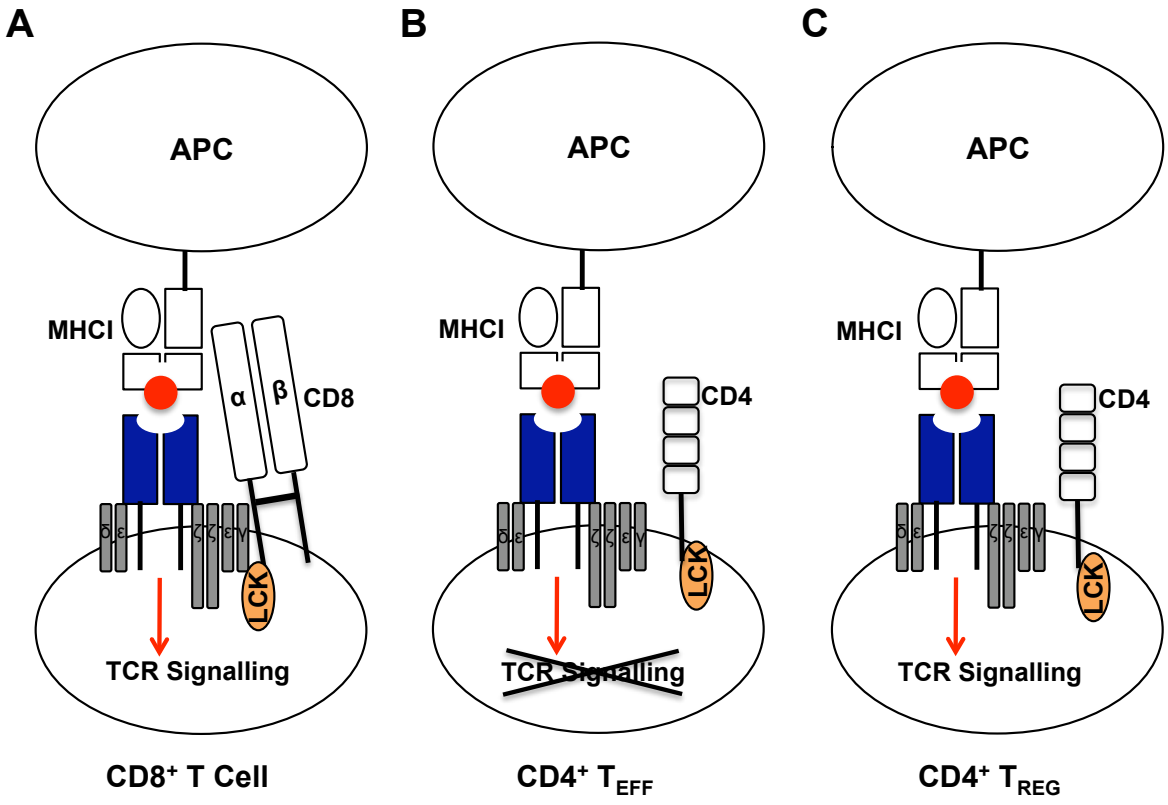

Figure 4-1 Model of The Function of MHCI restricted TCRs in Human T Cell Populations. (A) In a $CD8^+$ T cell an MHCI restricted TCR will recognise its specific pMHCI on the surface of an APC. The CD8αβ co-receptor stabilises this interaction and recruits the Lck tyrosine Kinase to the CD3 complex, which phosphorylates CD3 signalling domains, resulting in productive TCR signalling. (B) In a $CD4^+$ T_{EFF} cell, the CD4 co-receptor does not associate with MHCI to stabilise the interaction between the introduced MHCI TCR and pMHC interaction. Therefore, Lck is not recruited to the CD3 complex and productive TCR signalling does not occur. (C) In a $CD4^+$ T_{REG} cell, TCR signalling does not rely on CD4 recruitment of Lck. Therefore upon recognition of specific pMHCI by the MHCI restricted TCR, productive TCR signalling can occur. However, a mechanism for this has yet to be proposed.

4.2. Materials and Methods

Expansion of Transduced Primary Human T cell Populations.

Primary cells were isolated by FACS and activated following established protocols as described in section 2-1 (Brusko et al., 2010; Canavan et al., 2012; Skowera et al., 2008). Transduced T_{EFF} and T_{REG} cells were maintained until the end of their first expansion period, typically 8-10 days for T_{REG} and 10-12 days for T_{EFF} before a second round of cell expansion as described. After this second round of expansion, transduced T_{REG} and T_{EFF} were FACS sorted based on Rat CD2 expression. Depending on cell number, transduced cells were either re-expanded a third time or cryopreserved for future use. Where indicated, T_{REG} cells were additionally cultured in 100ng/ml Rapamycin (RAPA) (Rapammune, Pfizer Ltd, UK). Transduced $CD8^+$ T cells were cultured until D8, sorted based on Rat CD2 expression and then re-expanded using irradiated feeders at a ratio of 10:1 feeders:$CD8^+$ T cells with the addition of phytoheamagluttinin (PHA) at a final concentration of 5μg/ml. At the end of this round of expansion, transduced $CD8^+$ T cells were cryopreserved. Full timelines of the expansion protocol of the different primary T cell populations were as shown (Figure 4-2).

Activation assay for CD69 on Primary Human T Cell Populations

On Day 6 post initial activation of T cells, all cells were harvested and the magnetic aCD3/CD28 beads were removed using a 1.5 ml Eppendorf size magnet (Dynal, Life Technologies, UK). Samples were incubated on the magnet for 2 minutes and the beads washed a minimum of two times for 2 minutes to ensure maximal cell recovery. The cells were then washed at 400g for 5 minutes, re-suspended in IL-2 free XV5 and rested overnight to minimise background expression of CD69 in un-stimulated cultures. On Day 7 autologous PBMCs were thawed from liquid N_2, washed, labelled with Cell Trace Violet and re-suspended to $10x10^6$/ml. PBMCs were then pulsed with either 10μg of the peptide to be tested (SL9, ALW, RQF or VMN), 10μl PBS or 10μl CytoStim for 2 hours at 37°C in a shaking water bath. Cells were then washed in 4 ml XV5 at 400g for 5 minutes and re-suspended to $2x10^6$ cells/ml. Transduced cells were harvested and re-suspended to a

concentration of $4x10^5$/ml and the cells and PBMCs were added in a 1:1 mix to 96 u-bottom plates for a final PBMC:T cell ratio of 5:1. The cells were incubated at 37°C for 16 hours before being harvested and stained with fluorescently labelled antibodies against TCR specific vβ, Rat CD2 and CD69.

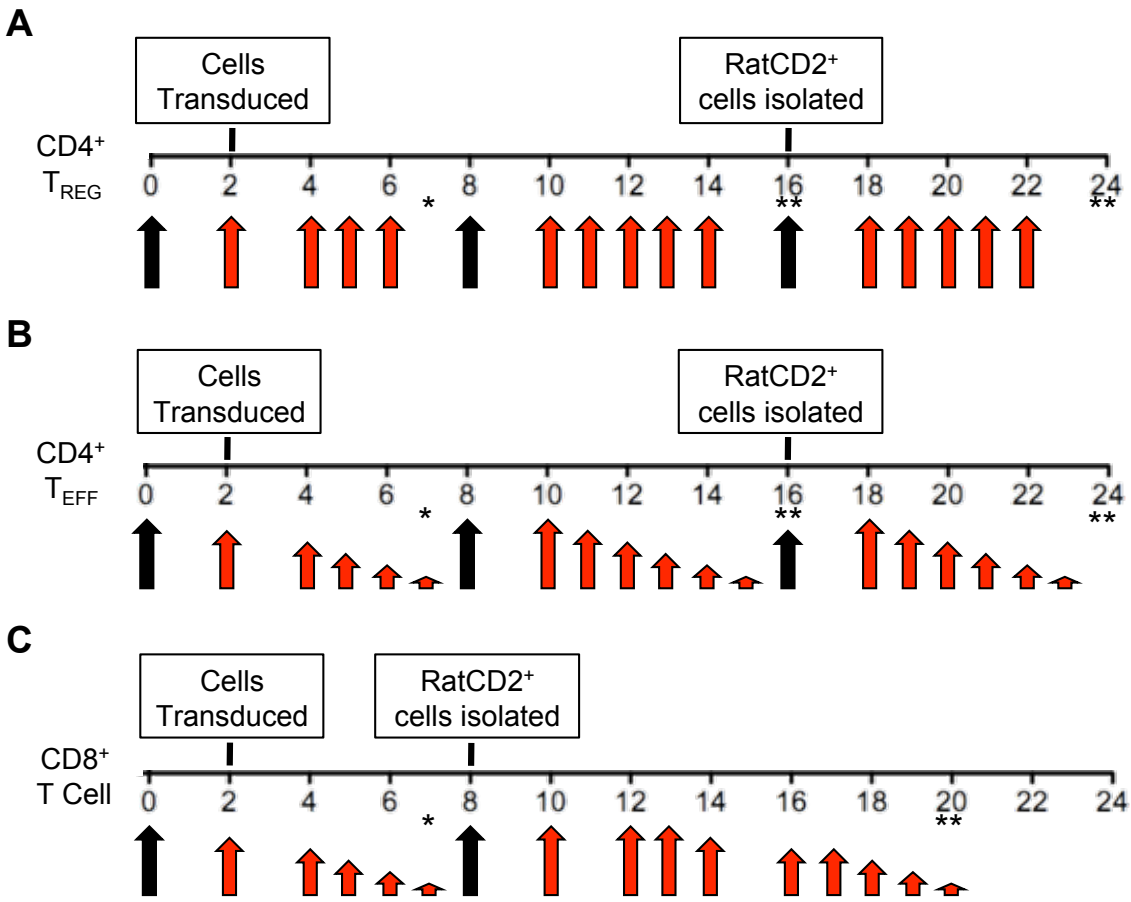

Figure 4-2 Expansion Protocol of Primary Human T Cell Populations. The timeline for initial activation, transduction and expansion of FACS isolated primary T cells populations is shown by day number for (A) $CD4^+$ T_{REG} cells, (B) $CD4^+$ T_{EFF} cells and (C) $CD8^+$ T cells. Black arrows indicate on which days cells were initially activated or activated for re-expansion. Red arrows indicate days where cells were split and given fresh IL-2 containing media. The red arrows decreasing in size indicate a halving in the amount of fresh IL-2 given to the cells. On D7 * denotes when cells were used in an activation assay and ** when cells were cryopreserved for later use.

Suppression of a Recall Response by Ag Specific T_{REG} Cells

Autologous PBMCs were thawed from liquid N_2 and labelled with Cell Trace Violet Stain. After wash steps, the cells were re-suspended to $2x10^6$/ml and added in 50μl to a 96 u-bottom plate for a final cell/well number of $1x10^5$. The Agrippal vaccine (Novartis, UK) is an inactivated influenza vaccine that contains haemagglutinin (HA) from three strains and will be referred to as HA throughout this thesis. It was used at a dilution of 1:500, a final concentration equivalent to 180ng/ml of HA, to stimulate the PBMCs. The CEF viral peptide mix (Mabtech, Sweden) was used at final concentrations equivalent to 10μM, 2.5μM and 1μM. Candida Albicans, strain number XPLM73, is a purified protein derivative (Greer Laboratories, North Carolina) and was used at final concentrations equivalent to 10μg/ml, 5μg/ml and 2μg/ml. The recall Ags HA, CEF and Candida were all used to test the memory T cell responses of each donor. HA was chosen for use in all suppression assays after eliciting the most robust proliferative responses from all donor PBMCs tested. Each peptide (SL9, ALW, RQF and VMN) used to stimulate the T_{REG} cells were added at 1μg/ml. Cryopreserved FACS sorted transduced T_{REG} cells were thawed, washed and re-suspended to $4x10^5$/ml to be added in 50μl to the wells. This allowed for a PBMC:T_{REG} ratio of 1:5. The assay was incubated at 37°C for 6 days before harvesting and staining with fluorescently labelled antibodies against CD3, CD4 and Rat CD2. The percentage suppression by the addition of peptide in the absence or presence of T_{REG} cell populations was calculated by ((% proliferation + HA - % proliferation HA + peptide) / % proliferation + HA) * 100.

4.3. Results

4.3.1. Isolation of Primary Human T cell Populations by FACS and T cell Activation

Purified populations of T cells were obtained using a FACS based approach (Figure 4-3). As cells were to be activated using αCD3/CD28 beads, detection of T cells using their definitive marker CD3 was avoided to ensure this signalling pathway would not be blocked by residual antibodies. To minimise contamination of isolated T cell populations, a dump channel was used. All cells were stained with CD14, CD16 and CD56 to minimise contamination of $CD4^+$ T cells with CD14 and CD16 expressing monocytes and $CD8^+$ T cells with $CD16^+CD56^+$ NK cells (Filion et al., 1990; Perfetto et al., 2004). $Dump^-CD4^-CD8^+$ cells were then isolated as the $CD8^+$ T cell population and $Dump^-CD4^+CD8^-$ cells were further separated into T_{REG} and T_{EFF} cell populations using expression of CD25 and CD127. It has been well established that Isolation of $CD4^+CD25^+CD127^{lo}$ cells yields a highly pure population of $CD4^+$ T_{REG} cells (Liu et al., 2006; Sakaguchi et al., 1995). Conversely, the $CD4^+CD25^{lo}CD127^{hi}$ population of cells identifies $CD4^+$ T_{EFF} cells (Cao et al., 2003). Therefore, in this study the $CD8^+$ T cell population refers to $Dump^-CD4^-CD8^+$ cells, the $CD4^+$ T_{EFF} population as $Dump^-CD4^+CD8^-CD25^{lo}CD127^{hi}$ cells and finally the $CD4^+$ T_{REG} population refers to cells that were $Dump^-CD4^+CD8^-CD25^+CD127^{lo}$ when isolated. LV vectors were first selected for gene therapy based on their ability to transduce non-dividing cells (Naldini et al., 1996). However, it has been shown that for effective transduction of T cell populations, including full integration and reverse transcription of introduced genes, the T cells are required to be in the G1b phase of cell cycle (Korin and Zack, 1998). Thus, the three primary T cell populations described were activated with αCD3/CD28 beads and cultured in various levels of IL-2 to permit cell expansion as detailed (Figure 4-2). $CD4^+$ T_{REG} cells were cultured in high exogenous levels of IL-2 and a bead to cell ratio of 1:1 in line with protocols used to expand large number of these cells (Earle et al., 2005; Putnam et al., 2009). The expansion potential of $CD4^+$ T_{REG} cells transduced with either the Mock, 868, 1E6 or 1D7 LV were measured over time in a single donor (Figure 4-4). This data was consistent with transduced $CD4^+$

T_{REG} cells from other donors that were transduced and expanded following the same protocols (data not shown).

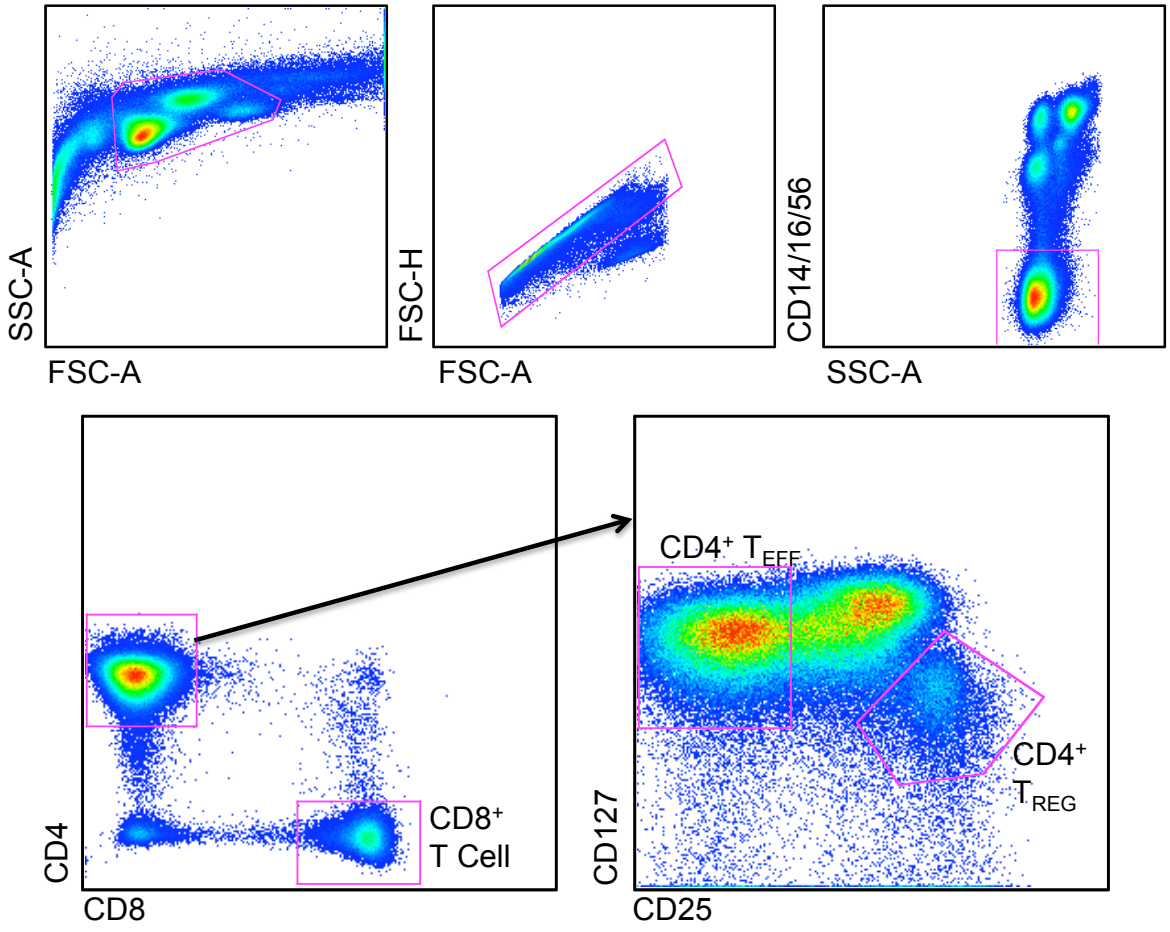

Figure 4-3 FACS Isolation Strategy of Primary Human T Cell Populations. PBMCs from an HLA-A2$^+$ donor were isolated and stained with fluorescently labelled antibodies. Live cells were selected on their FSC-A/SSC-A profile and doublets excluded based on FSC-A/FSC-H. A dump channel was used to exclude any CD14$^+$, CD16$^+$ and CD56$^+$ cells. CD8$^+$ cells were then isolated and CD4$^+$ cells separated into CD127^{hi}CD25^{lo} T_{EFF} and CD127^{lo}CD25$^+$ T_{REG} cells.

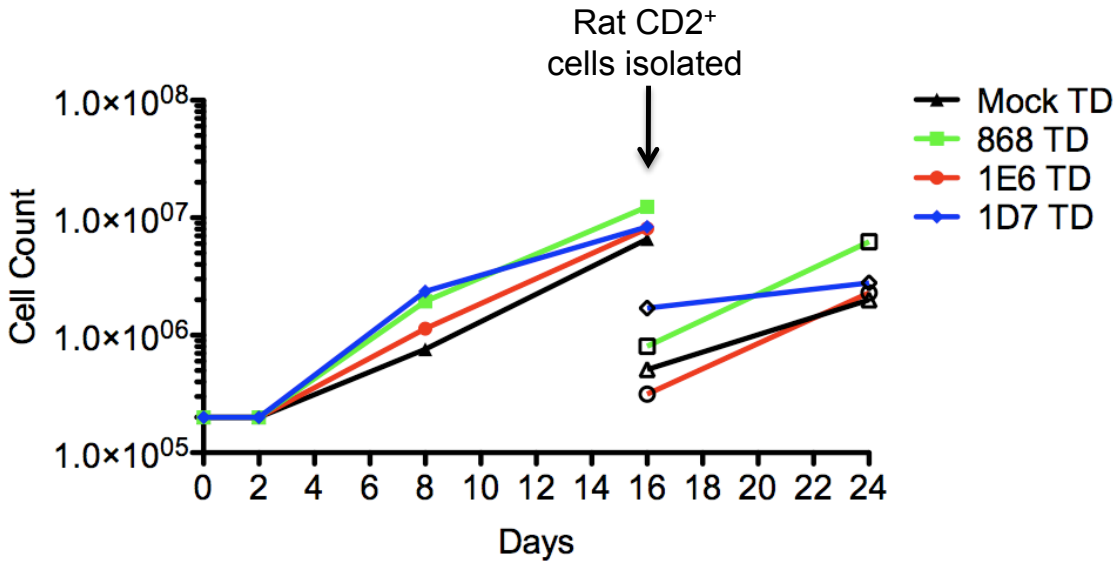

Figure 4-4 Expansion of LV Transduced CD4$^+$ T_{REG} cells over time. The graph shows whole population cell counts of transduced T_{REG} cells through three rounds of expansion Cells were sorted based on Rat CD2 expression on D16 and expanded for a further 10 days. Data shown is from a T_{REG} expansion from a single blood donor and is representative of other T_{REG} expansions from different donors.

4.3.2. Human T cells can be transduced with LV and exhibit differential expression pattern of introduced TCRs.

Primary human T cell populations were transduced with LV vectors 48 hours post their initial activation, in line with established protocols (Brusko et al., 2010). During the course of these studies, n=12 transduction were performed using cells from four HLA-A2$^+$ donors. The transduction efficiency of all cell population were assessed on the basis of Rat CD2 expression. Analysis of this data using an analysis of variance statistical test identified a statistically significant difference between the transduction efficiency of the three T cell populations ($p<0.01$). Further analysis identified that transduction was significantly higher for CD4$^+$ T_{REG} (mean 37.5 ± 17.1% SD) than either CD4$^+$ T_{EFF} (mean 22.6 ± 11.9% SD) or CD8$^+$ T cells (mean 17.3 ± 7.9% SD) (Figure 4-5).

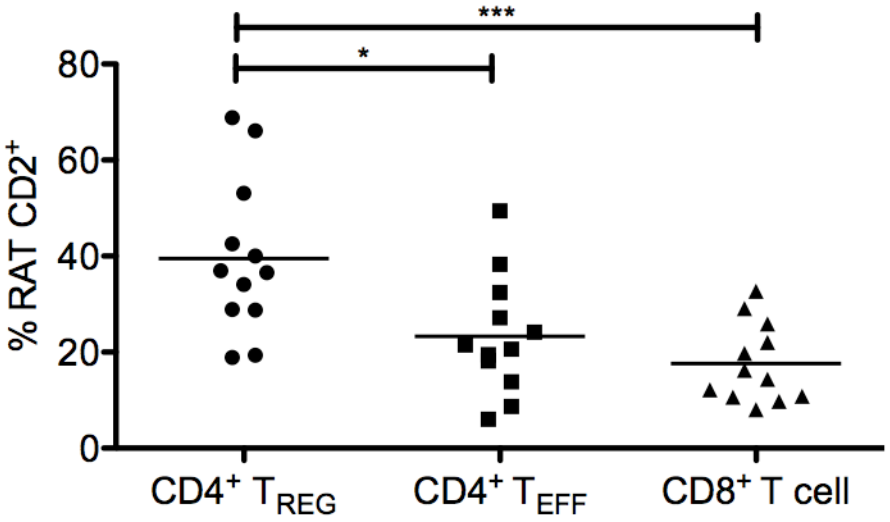

Figure 4-5 CD4$^+$ T_{REG} cells are more readily transduced that CD4$^+$ T_{EFF} and CD8$^+$ T cells. The transduction efficiency of CD4$^+$ T_{REG}, CD4$^+$ T_{EFF} and CD8$^+$ T cells was assessed by expression of the transduction marker Rat CD2. Plot shows the percentage transduction of cells from four blood donors that were transduced with either the 868 LV, 1E6 LV or 1D7 LV. Paired Student's t tests were used to calculate data significance p value * = <0.05 and p value *** = <0.001

When comparing transduction of the three cell populations from a single individual with the 868 TCR a clear dual expression of the 868 clonotypic TCR vβ5 chain and Rat CD2 marker was observed in all three cell populations (Figure 4-6). This was in stark contrast to when cells were transduced with the autoreactive 1E6 and 1D7 TCRs (Figure 4-7). Specifically, not all cells that were transduced with the 1E6 LV, as evidenced by Rat CD2 expression, were positive for expression of the 1E6 clonotypic vβ chain, vβ8 (Figure 4-7(A)). This pattern was observed in all three cell populations was similar to transduction with the 1D7 TCR and its specific clonotypic vβ chain, vβ2 (Figure 4-7 (B)).

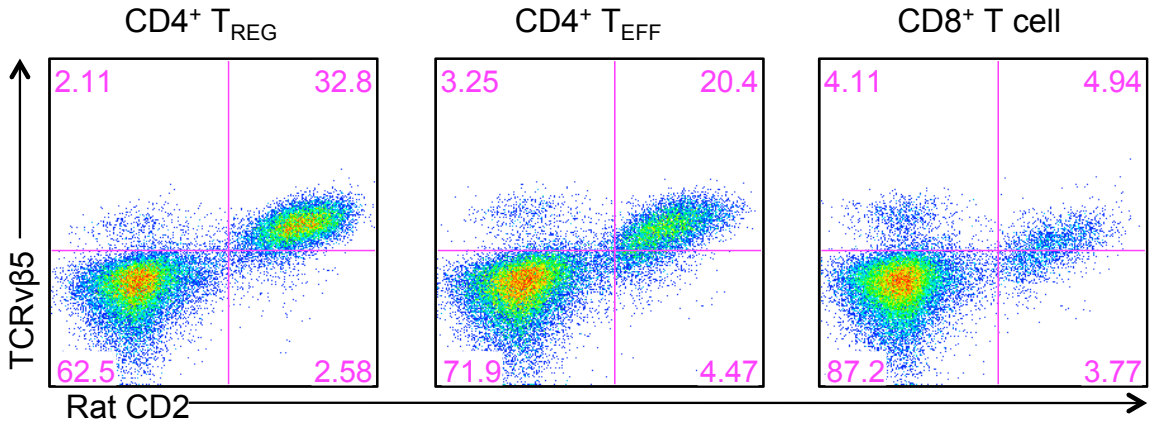

Figure 4-6 Transduction with the 868 Pathogen Specific TCR. $CD4^+$ T_{REG}, $CD4^+$ T_{EFF} and $CD8^+$ T Cells were transduced with LV encoding the 868 TCR and successful transduction was assessed by staining transduced cell populations with αTCRvβ5 and αRat CD2 antibodies.

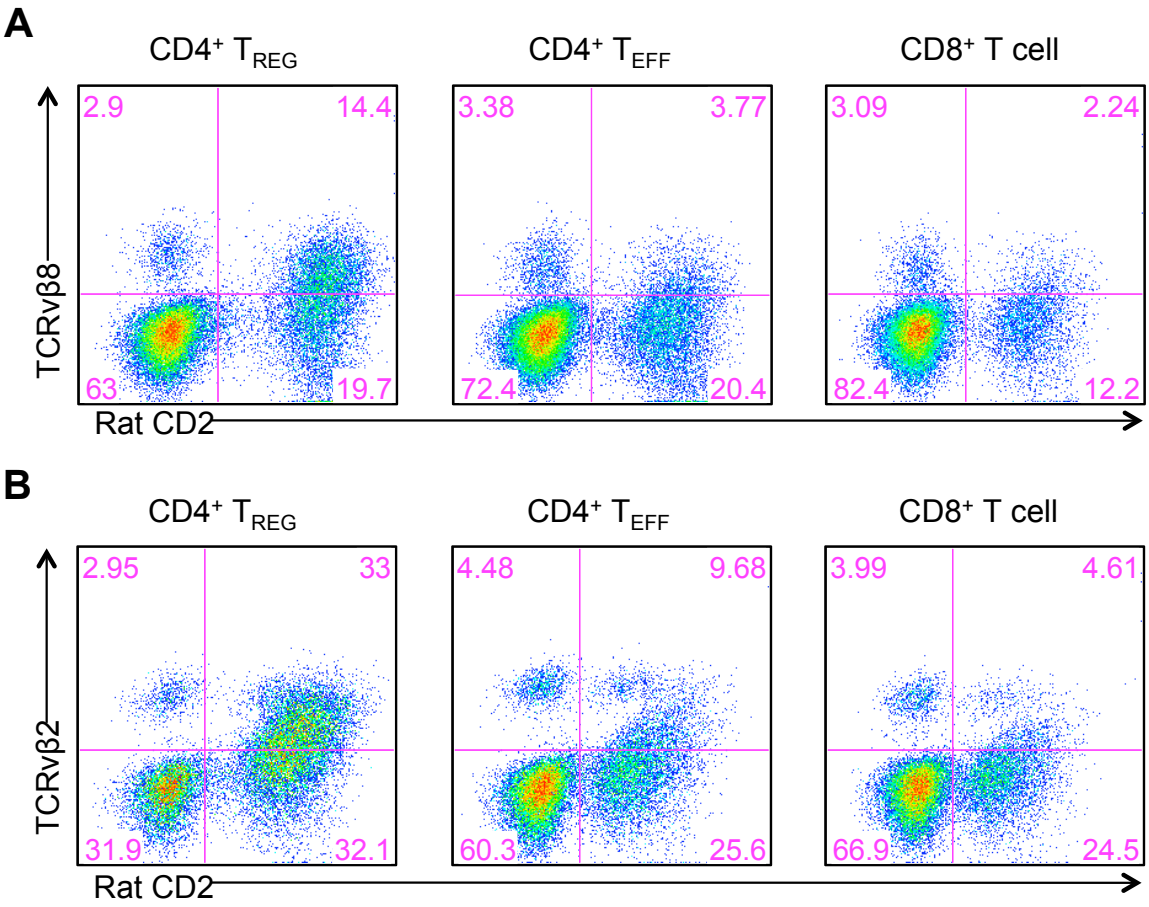

Figure 4-7 Expression of Transduced Autoreactive TCRs in Primary Human T Cell Populations. $CD4^+$ T_{REG}, $CD4^+$ T_{EFF} and $CD8^+$ T cells were transduced with LV encoding autoreactive TCRs. Representative FACS plots show (A) cell populations transduced with the 1E6 TCR and stained with αTCRvβ8 and αRat CD2 antibodies, (B) and cell populations transduced with the 1D7 TCR and stained with αTCRvβ2 and αRat CD2 antibodies.

A further difference between the expression of the pathogen specific TCR and autoreactive TCRs was identified when the MFI of the endogenous vβ chain vs. the introduced vβ chains on $CD4^+$ T_{REG} cells was assessed (Figure 4-8). The expression of the endogenous vβ5 of the 868 was comparable to expression of introduced vβ5 (mean 760 and 958 respectively) (Figure 4-8 (A)). Although not significant, there was a definite trend of a higher expression of endogenous vβ8 compared to introduced 1E6 vβ8 (mean 892 vs. 684) (Figure 4-8 (B)). Again, this

trend for higher expression of the endogenous vβ chain was seen for vβ2 expression vs. the introduced 1D7 Vβ2 (mean 2444 vs. 1170) (Figure 4-8 (C)).

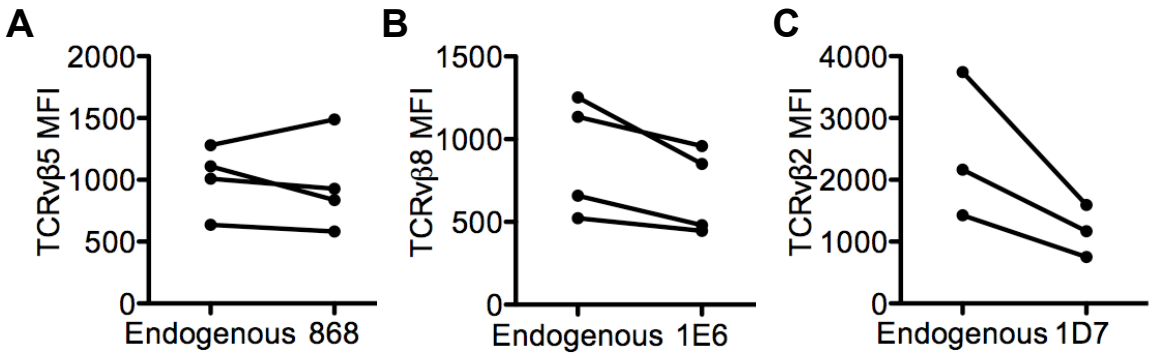

Figure 4-8 The Expression of Autoreactive TCRs Tends to be Lower Than The Expression of Endogenous TCR. Transduced populations of T_{REG} cells were stained for the expression of the clonotypic vβ of the introduced TCR. The cells were then divided into non-transduced Rat $CD2^-$ and transduced Rat $CD2^+$ cells and the MFI of the clonotypic vβ was assessed as being endogenous TCR and Introduced TCR respectively for (A) 868 vβ5 (B) 1E6 vβ8 and (C) 1D7 vβ2 expression. Each pair represents a population of cells from an individual donor.

4.3.3. Transduced T Cells Retain Their Phenotype at D8 Post Expansion

After the first round of expansion, the phenotype of transduced cells was assessed by flow cytometry to determine the purity of expanded populations. To this end, transduced primary T cell populations were stained with fluorescently labelled antibodies against CD3, CD4 and CD8. Using this panel of antibodies, it can be seen from the representative FACS plots that on D8 the $CD4^+$ T_{REG} cells were > 97% pure, the $CD4^+$ T_{EFF} cells > 99% pure and $CD8^+$ T cells >95% pure (Figure 4-9 (A)). It is well established, that constitutive high-level expression of the transcription factor FoxP3 denotes a stable human $CD4^+$ T_{REG} cell (Roncador et al., 2005). However, it has also been shown that this marker can be transiently expressed within $CD4^+$ T_{EFF} cells (Gavin et al., 2006). Therefore, intracellular staining for FoxP3 expression was performed on the expanded transduced cells, along with co-staining for CD25. Importantly, all cells were starved of IL-2 for 48

hours prior to FoxP3 staining to minimise expression of FoxP3 due to transient up-regulation via the IL-2 pathway (Zorn et al., 2006). From the representative FACS plots shown, it is clear that stable high expression of FoxP3 was only found in the expanded $CD4^+$ T_{REG} cells (Figure 4-9 (B)). Although there was moderate co-expression of FoxP3 and CD25 in both $CD4^+$ T_{EFF} and $CD8^+$ T cells, this was consistently at a lower percentage and MFI compared to $CD4^+$ T_{REG} cells (Figure 4-9 (B)). These data demonstrate that transduction and expansion of the individual T cell populations had no gross effect on the phenotype of these cells, as judged by CD3, CD4, CD8 and FoxP3 expression.

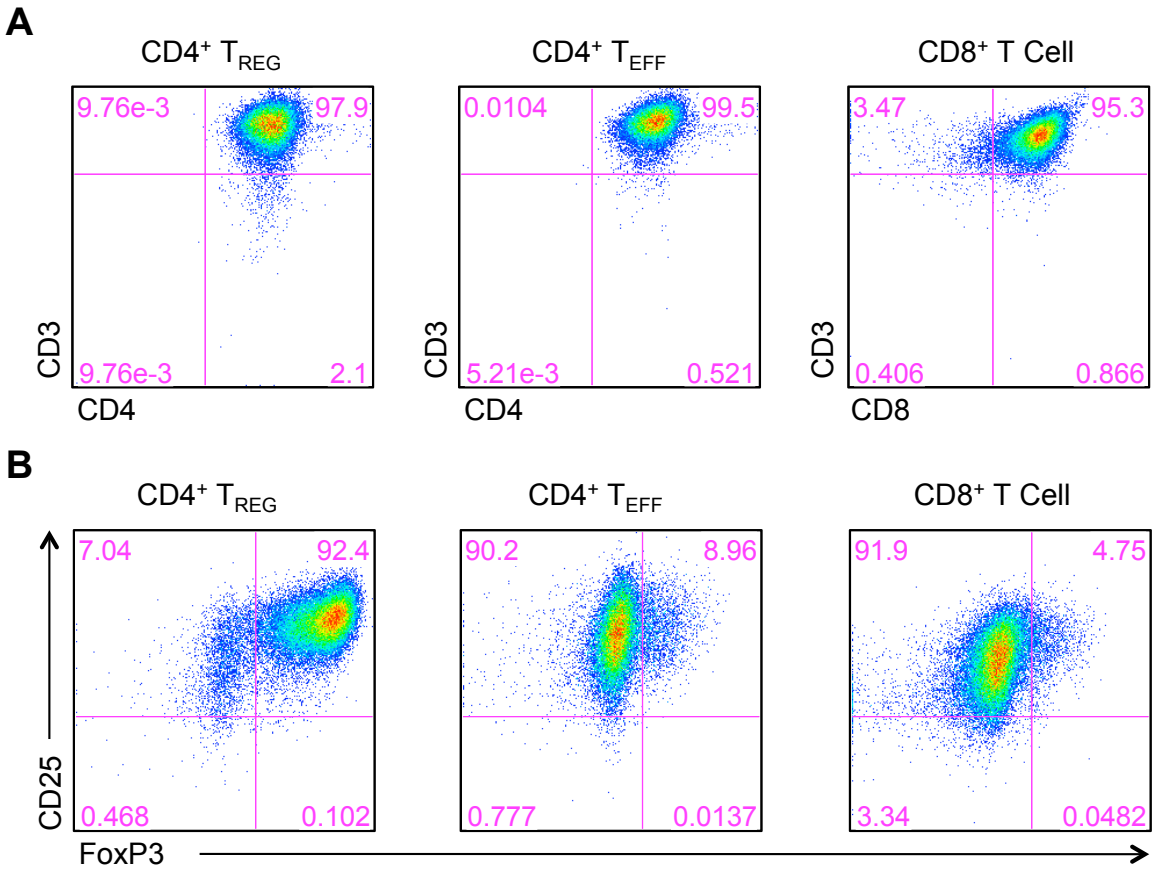

Figure 4-9 Phenotype of Transduced and Expanded Cells. The phenotypes of transduced $CD4^+$ T_{REG}, $CD4^+$ T_{EFF} and $CD8^+$ T cells were assessed on D8 post initial activation. (A) Representative FACS plots show surface phenotype stains of $CD4^+$ T_{REG} and T_{EFF} cells stained with αCD3 and αCD4 fluorescent antibodies and $CD8^+$ T cells stained with αCD3 and αCD8 antibodies. (B) Representative FACS plots show cell populations stained for expression of CD25 and FoxP3.

4.3.4. High Affinity 868 TCR can effectively Signal in All T Cell Populations Tested

To determine whether the *de novo* expressed TCRs could signal in all three cells populations, CD69 up-regulation, as a marker of T cell activation, was assessed in response to specific peptide. This assay had a similar set up to the Jurkat activation assay used in Chapter 3-3 and for each donor tested, autologous Violet labelled PBMCs were used as the APCs. In the Jurkat activation assay, sorted populations of TCR transduced cell lines were used to look at the response of only the TCR expressing cells. However, with the transduced primary T cells, the fact that there were four populations of cells, delineated by their TCRvβ and Rat CD2 expression within the whole cell population, provided useful internal controls for each experiment. Taking the 868 TCR transduced cells as an example; the presence of Rat $CD2^-$ cells could be used as an internal negative control to ensure any CD69 up-regulation in response to SL9 peptide was specifically in the 868 (Rat $CD2^+$) transduced population (Figure 4-10 (A)). Moreover, by separating endogenous TCRvβ5 expressing cells in the non-transduced population, provided a control for the effect of Vβ5 expression on CD69 up-regulation in response to SL9 peptide. All transduced cell populations were then stimulated with autologous Violet labelled PBMCs that had been pulsed with PBS, as a negative control, CytoStim as a positive control, or the specific peptide for the transduced population (Figure 4-10 (B)).

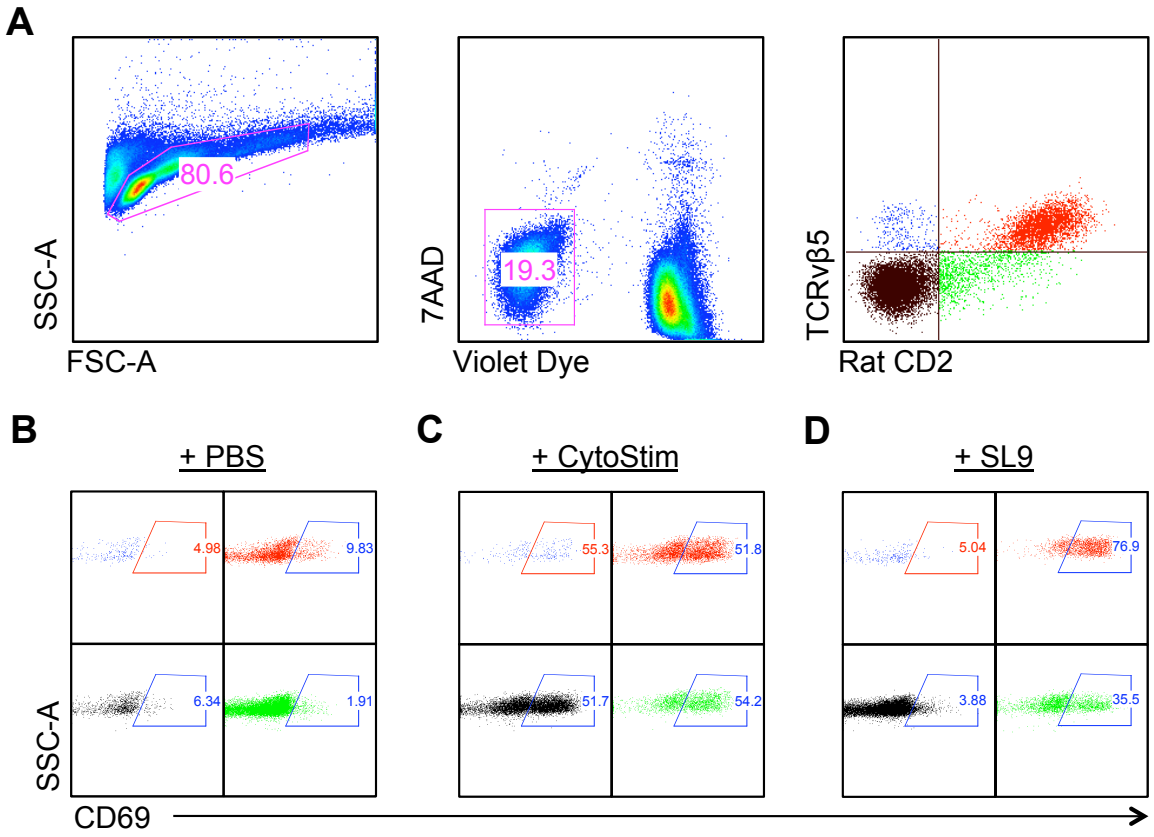

Figure 4-10 Gating Strategy for CD69 Expression on Peptide Stimulated Transduced Cells. Transduced T Cell lines were incubated with autologous Violet labelled PBMCs pulsed with PBS, CytoStim or specific peptide on D8 post initial activation (A) Representative FACS plots show gating strategy used for 868 Transduced T cells. Live cells were gated based on their FSC-A/SSC-A profile and then Transduced T Cells positively gated on to remove Violet labelled PBMCs and 7AAD$^+$ dead cells. Cells were also stained with specific vβ antibody of the introduced TCR and Rat CD2 to split cells into non-transduced TCRvβ5$^-$Rat CD2$^-$ cells (Black) and endogenous vβ expressing TCRvβ5$^+$Rat CD2$^-$ (blue) cells and Transduced TCRvβ5Rat CD2$^+$ (red) and TCRvβ5$^-$Rat CD2$^+$ (green) cells. The CD69 expression on each population was then assessed for cells stimulated with (B) PBS (C) CytoStim and (C) SL9.

To first test whether the 868 TCR could effectively signal in CD4$^+$ T_{REG} cells the 868 TCR transduced T_{REG} cells were stimulated with PBMCs pulsed with PBS, CytoStim or SL9 peptide. Using the gating strategy shown (Figure 4-11 (A)) the level of CD69 up-regulation in the four populations of T_{REG} cells was ascertained. In keeping with results observed in TCRαβ deficient Jurkat cell lines in Chapter 3-

3, cells transduced with the 868 TCR significantly up-regulated CD69 in response to the SL9 peptide pulsed PBMCs and not in response to PBS pulsed control PBMCs (Figure 4-11 (B). Moreover, this CD69 up-regulation was specific to the Rat CD2 transduced population. Interestingly up-regulation of CD69 was also observed in vβ5$^-$RatCD2$^+$ cells. As expected, the same pattern of activation was seen in both 868 transduced CD4$^+$ T_{EFF} and CD8$^+$ T cells (Figure 4-11 (C-D)).

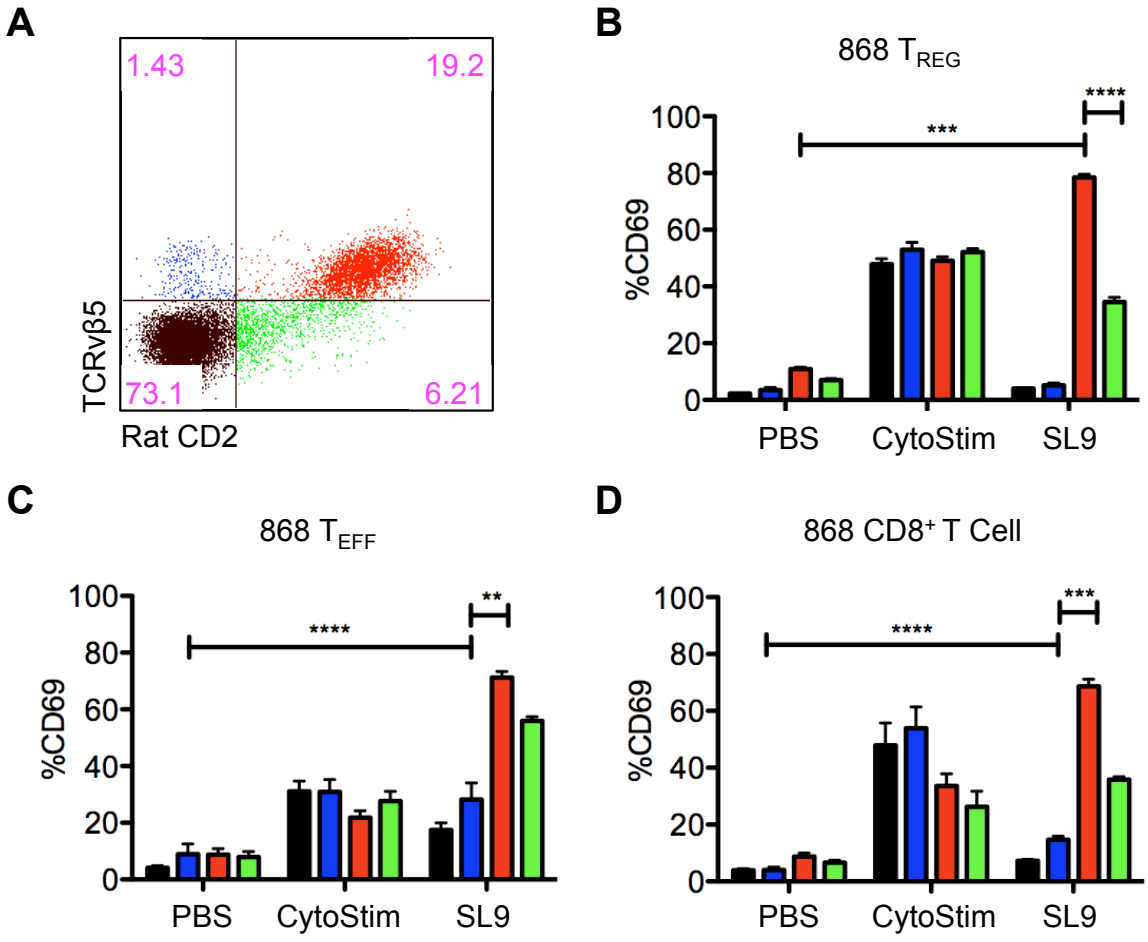

Figure 4-11 Activation of 868 Transduced T Cell lines. (A) Representative FACS plot of CD4$^+$ T_{REG} cells showing the gating strategy used to identify non-transduced TCRvβ5$^-$Rat CD2$^-$ cells (Black) and endogenous vβ expressing TCRvβ5$^+$Rat CD2$^-$ (blue) cells as well as transduced TCRvβ5$^+$Rat CD2$^+$ (red) and TCRvβ5$^-$Rat CD2$^+$ (green) cells. Graphs show percentage CD69 on four population of activated 868 transduced (B) CD4$^+$ T_{REG} (C) CD4$^+$ T_{EFF} and (D) CD8$^+$ T cells. Data shown is for one donor and three replicates in a single assay and is representative of a further three blood donors. An unpaired Student's t test was used to calculate data significance. P Value = ** <0.01 *** < 0.001 **** <0.0001.

4.3.5. Signalling capabilities of autoreactive MHCI TCRs in Human T Cell Populations.

The ability of the autoreactive TCR transduced T cells to signal in response to peptide were assayed in the same way as the 868 transduced T cells. The 1E6 transduced T cells were stimulated with PBS, CytoStim, ALW cognate peptide or the super agonist peptide RQF pulsed PBMCs. For analysis of CD69 up-regulation, the transduced cells were delineated into 4 populations based on TCRvβ8 and Rat CD2 expression (Figure 4-12 (A)). Similar to results seen with the Jurkat cells, $CD4^+$ T_{REG} cells transduced with the 1E6 TCR failed to respond to the ALW peptide (Figure 4-12 (B)). The transduced cells however did respond to the super agonist RQF peptide showing that this TCR can signal in T_{REG} cells. This response was observed only in the Rat CD2 expressing population and only in response to the RQF peptide. Interestingly, the $TCRv\beta8^-RatCD2^+$ cells were capable of responding to the RQF peptide, although this response was of a lower magnitude than the $TCRv\beta8^+RatCD2^+$ cells (Figure 4-12 (B)). Not surprisingly, the $CD4^+$ T_{EFF} cells transduced with the 1E6 TCR were also unable to up-regulate CD69 in response to the ALW peptide (Figure 4-12 (C)). However, it was of interest to note that 1E6 transduced $CD8^+$ T cells also failed to respond to the cognate ALW peptide, despite the presence of the CD8αβ co-receptor (Figure 4-12 (C)). Additionally, in all three cell populations tested, cells transduced with the 1D7 TCR were incapable of signalling in response to the cognate VMN peptide (Figure 4-13).

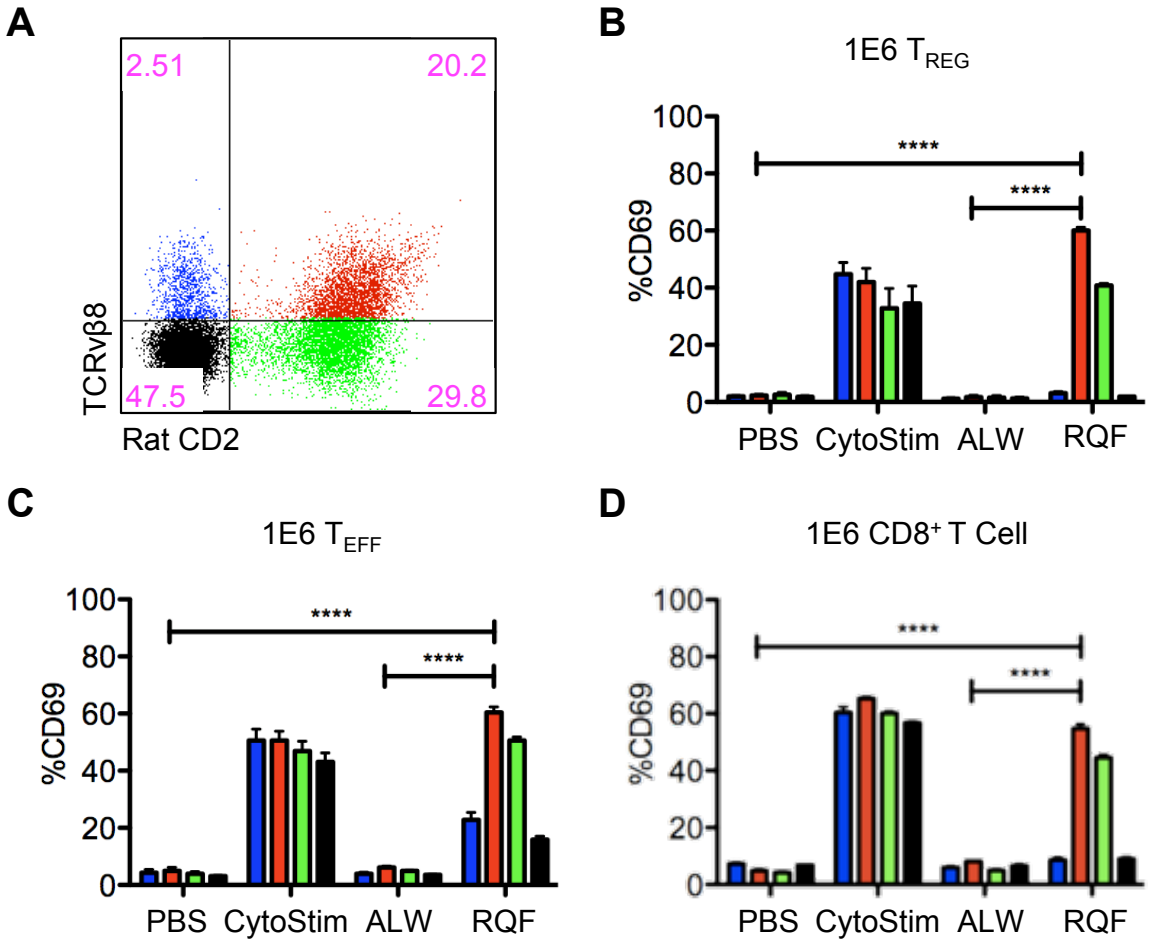

Figure 4-12 Activation of 1E6 Transduced T Cell lines. (A) Representative FACS plots of $CD4^+$ T_{REG} cells showing the gating strategy used to identify non-transduced $TCRv\beta8^-$Rat $CD2^-$ cells (Black) and endogenous vβ expressing $TCRv\beta8^+$Rat $CD2^-$ (blue) cells as well as transduced $TCRv\beta8^+$Rat $CD2^+$ (red) and $TCRv\beta8^-$Rat $CD2^+$ (green) cells. Graphs show percentage CD69 expression on the four population of activated 1E6 transduced (B) $CD4^+$ T_{REG} (C) $CD4^+$ T_{EFF} and (D) $CD8^+$ T cells. Data shown is for one donor and three replicates in a single assay and is representative of a further three blood donors. An unpaired Student's t test was used to calculate data significance. P Value = **** <0.0001.

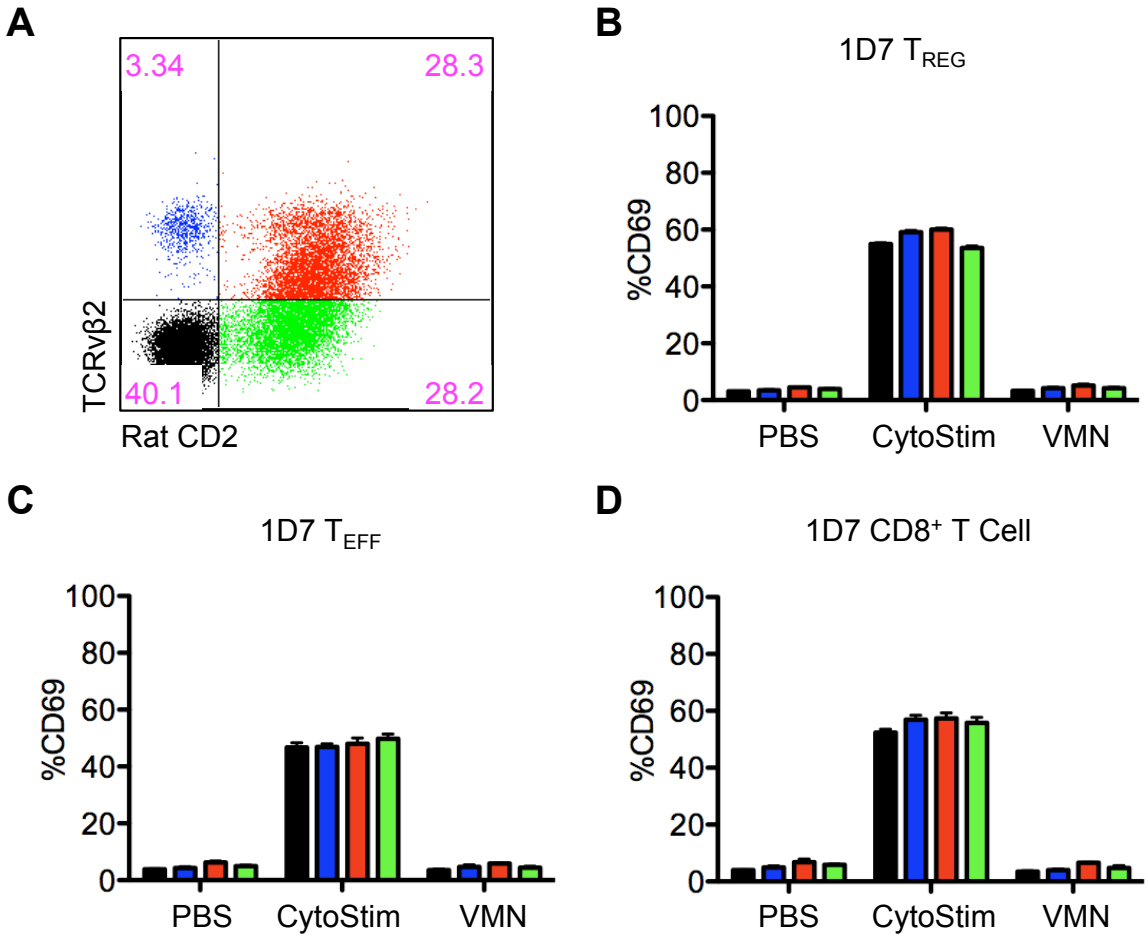

Figure 4-13 Activation of 1D7 Transduced T Cell lines. (A) Representative FACS plot of CD4$^+$ T_{REG} cells showing the gating strategy use to identify non-transduced TCRvβ8$^-$Rat CD2$^-$ cells (Black) and endogenous vβ expressing TCRvβ2$^+$Rat CD2$^-$ (blue) cells as well as transduced TCRvβ2$^+$Rat CD2$^+$ (red) and TCRvβ2$^-$Rat CD2$^+$ (green) cells. Graphs show percentage CD69 expression on the four population of activated 1D7 transduced (B) CD4$^+$ T_{REG} (C) CD4$^+$ T_{EFF} and (D) CD8$^+$ T cells. Data shown is for one donor and three replicates in a single assay and is representative of a further three blood donors.

4.3.6. Optimisation of an Ag Specific Suppression Assay to Measure MHCI TCR Function in Human T_{REG} Cells.

Currently the ability of the MHCI restricted TCRs to signal in $CD4^+$ T_{REG} cells have only been assessed via the up-regulation of CD69. However, there is no evidence to suggest that the up-regulation of CD69 in response to peptide correlates with suppressive function. Therefore, an Ag specific suppression assay was designed to measure whether the introduced TCR could confer Ag suppressive capabilities to a $CD4^+$ T_{REG} cell. In order to have a standard responding population that all transduced T_{REG} cells would be able to suppress, a recall Ag was chosen that could be used to stimulate PBMCs. Additionally, as discussed T1D pathogenesis is characterised by the effector functions of memory $CD4^+$ and $CD8^+$ T cells and therefore the ability of Ag specific T_{REG} cells to suppress a memory T cell response is vital (Roep and Peakman, 2011). The Ag required, therefore would have to be capable of stimulating robust responses in $CD4^+$ and $CD8^+$ T cells in multiple donors. The recall Ags of choice were; Agrippal - an inactivated surface Ag influenza vaccine that will be referred to as HA, Candida Albicans a purified protein derivative, and CEF, a viral peptide mix. To minimise the potential for activation of $CD4^+$ T_{REG} cells due to alloreactivity, an autologous system was used. PBMCs were Violet labelled and stimulated with three doses of each Ag and the proliferation of $CD4^+$ ($CD3^+CD4^+$) and $CD8^+$ ($CD3^+CD4^-$) T cells (Figure 4-14) was analysed. In the two donors examined, the HA Ag gave the most robust and reproducible proliferative response from both $CD4^+$ and $CD8^+$ T cells and was chosen as the Ag for all suppression assays to follow. The assay schematic is detailed in Figure 4-15 (A) and using this set up both “non-Ag specific” suppression, when $CD4^+$ T_{REG} cells were present in the culture but not activated through their introduced TCR, and Ag specific suppression could be measured. Only sorted Rat $CD2^+$ cells were used in the suppression assays, to exclude these cells, based on the expression of this marker, from the analysis of proliferating $CD4^+$ T cells (Figure 4-15 (B)).

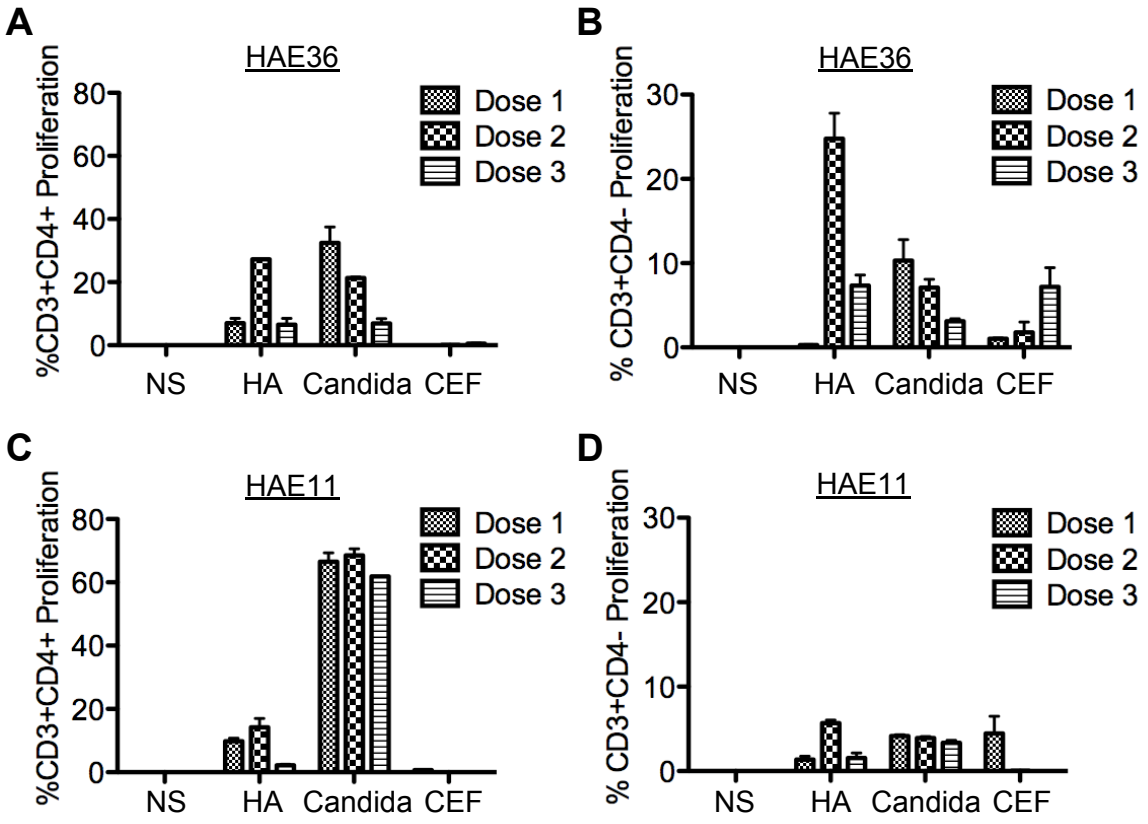

Figure 4-14 Optimisation of Stimulation Dose for Suppression Assay. Violet labelled PBMCs from two donors, HAE36 and HAE11 were stimulated with three doses of HA Ag, Dose 1 – 1/50, Dose 2 – 1/500, Dose 3 – 1/5000, 3 doses of Candida, Dose 1 - 10μg/ml, Dose 2 - 5μg/ml, and Dose 3 –2μg/ml and 3 doses of CEF Peptide pool, Dose 1 – 10μM, Dose 2 – 2.5μM Dose 3 – 1μM. Proliferation was then measured 6 days later by assessing Violet dye dilution of (A) HAE36 $CD3^+CD4^+$ and (B) HAE36 $CD3^+CD4^-$ T cells as well as (C) HAE11 $CD3^+CD4^+$ and (D) HAE11 $CD3^+CD4^-$ T cells.

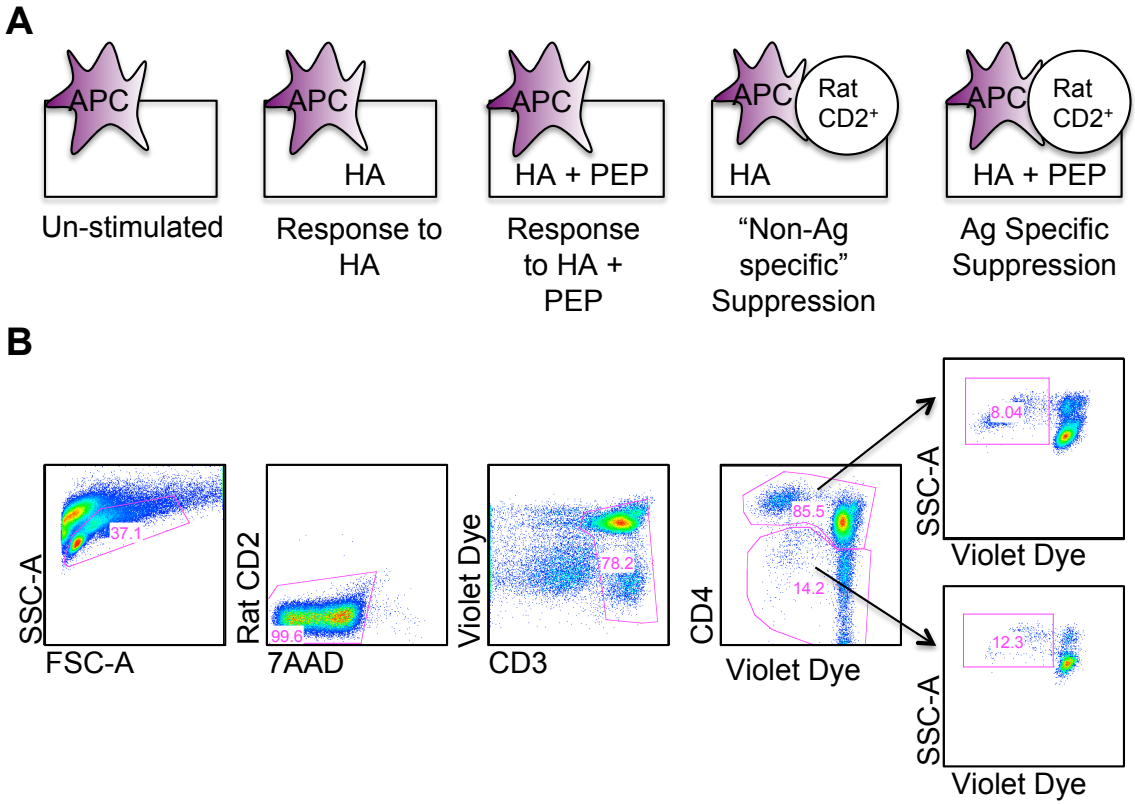

Figure 4-15 Schematic Representation and Gating Strategy for Ag Specific Suppression Assay. (A) Schematic shows the set up for the Ag specific suppression assay. Violet labelled autologous PBMCs were either un-stimulated, stimulated with HA recall Ag or stimulated with HA and specific peptide (PEP) for 6 days. A reduction in proliferation when TCR transduced T_{REG} cells were present in culture, but not stimulated with their specific peptide, was referred to as "non-Ag specific" suppression. A reduction in proliferation when TCR transduced T_{REG} cells were present in the culture and stimulated by their specific peptide was referred to as Ag specific suppression. (B) Representative FACS plots show the gating strategy used for the analysis of this assay. Lymphocytes were identified based on their FSC-A/SSC-A profile. Dead cells and transduced T_{REG} cells were excluded based on their expression of 7AAD and Rat CD2. Responder T cells were identified as $CD3^{+}$`Violet Dye^{+} and the proliferation in responding $CD4^{+}$ and $CD8^{+}$ subsets then examined by assessing the loss of Cell Trace Violet Dye in $CD3^{+}CD4^{+}$ and $CD3^{+}CD4^{-}$ cells respectively.

4.3.7. 868$^+$ TCR Transduced T_{REG} Cells exhibit Ag Specific suppression.

To be able to assess the functional capabilities' of the TCR transduced T_{REG} cells the phenotype of these cells was determined to ensure the stability of the CD4$^+$ T_{REG} cell markers. The cells used in these assays had been sorted based on their expression of Rat CD2 on D16 and expanded with αCD3/CD28 beads a third time before being cryopreserved at the end of the third cycle of expansion. The cells were thawed and the phenotype of transduced 868$^+$ T_{REG} and 1E6$^+$ T_{REG} cells was ascertained. Co-staining with the clonotypic vβ and Rat CD2 antibodies confirmed these cells were >95% Rat CD2$^+$ (Figure 4-16 (A-B)). Additionally these cells were stained for intracellular expression of FoxP3, and although some of these cells had lost FoxP3 expression they remained >80% FoxP3$^+$ (Figure 4-16 (C)). Data shown is from expanded T_{REG} cells from donor HAE36 however similar results were seen after expansion of transduced T_{REG} cells from donor HAE11.

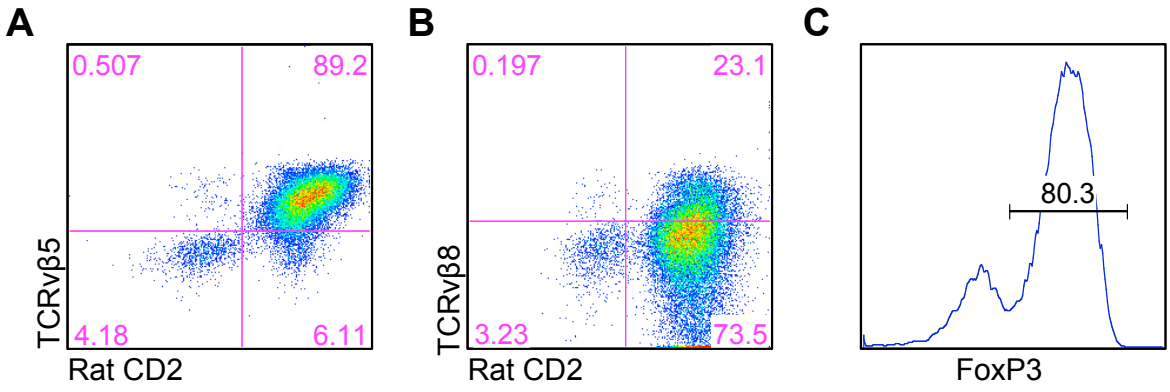

Figure 4-16 Phenotype of Expanded Transduced T_{REG} for use in Ag Specific Suppression Assay. Transduced T_{REG} cells from donor HAE36 that had been expanded for 16 days, sorted based on their Rat CD2 expression and expanded for a further 8 days were used in the suppression assay. Representative FACS plots show the phenotype of (A) 868$^+$ T_{REG} and (B) 1E6$^+$ T_{REG}. (C) A representative histogram shows the FoxP3 expression of the cells when they were used in the suppression assay.

The following results are shown using transduced and expanded T_{REG} cells and autologous PBMCs from Donor HAE36. In the absence of T_{REG} cells, a robust proliferative response by HAE36 $CD4^+$ T cells to the HA Ag was observed. This proliferative response was not affected by the presence of SL9 peptide (Figure 4-17 (A&D)). Mock T_{REG} cells, that were transduced but only express Rat CD2, were used as a control for the presence of T_{REG} cells. Upon addition of either Mock T_{REG} cells, or 868^+ T_{REG} cells, at a ratio of 5 PBMCs to 1 T_{REG} cell, a marked reduction in the proliferation of HA responding $CD4^+$ T cells was observed. This reduction in proliferation was comparable between the addition of Mock or 868^+ T_{REG} cells (Figure 4-17 (A-C)) and represents "non-Ag specific" suppression. When 868^+ T_{REG} cells were stimulated by the SL9 peptide, HA specific $CD4^+$ T cell proliferation was further reduced (Figure 4-17 (C&F)). However, no such reduction was seen when Mock T_{REG} cells were stimulated with SL9 (Figure 4-17 (B&E)). These results demonstrate that 868^+ T_{REG} cells are capable of mediating antigen specific suppression when stimulated with SL9 peptide. A similar pattern of proliferation was observed for HAE36 $CD8^+$ T cell responses, with addition of both Mock and 868^+ T_{REG} cells leading to a significant reduction in HA specific proliferation in the absence of SL9 peptide (Figure 4-18 (A-C)). A further reduction in proliferation was observed upon stimulation of 868^+ T_{REG} cells (Figure 4-18 (C&F)), but not Mock T_{REG} cells (Figure 4-18 (B&E)), with SL9 peptide. The FACS plots in Figures 4-17 and 4-18 represent pooled triplicate wells for each culture condition. Combined analysis of three independent triplicates for each culture condition are shown in Figure 4-19 (A&B) and demonstrate a reproducible and significant reduction in $CD4^+$ and $CD8^+$ T cell proliferation when 868^+ T_{REG} cells were stimulated with SL9 peptide. Ag specific suppression was quantified by calculating the percent reduction in proliferation upon the addition of SL9 peptide in the presence or absence of a particular T_{REG} cell population (Figure 4-19 (C&D)). These results were confirmed in a second completely independent experiment using transduced T_{REG} cells and autologous PBMC from HAE11 (Figure 4-19 (E&F)). These data demonstrate that for optimal suppression of $CD4^+$ and $CD8^+$ T cells, stimulation of 868^+ T_{REG} cells with specific Ag is required.

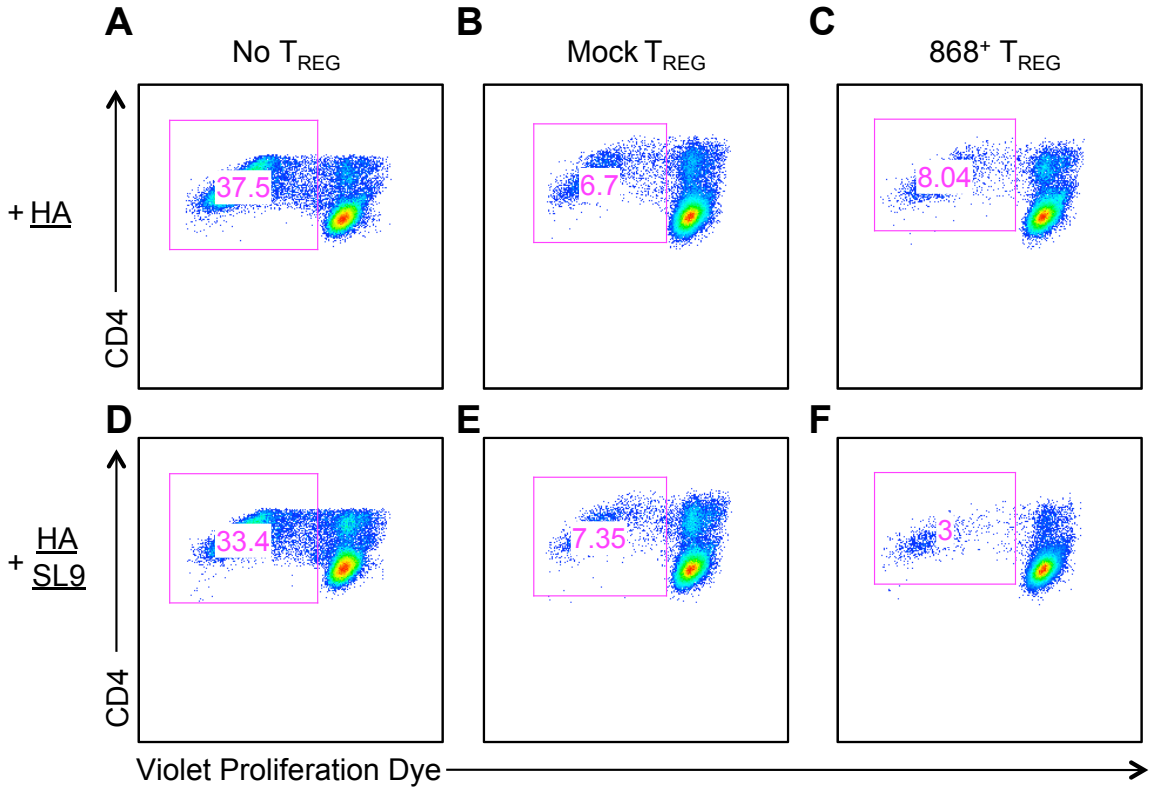

Figure 4-17 FACS plots Showing A Reduction of $CD4^+$ T cell Proliferation by Activated 868^+ T_{REG} Cells. Violet labelled PBMCs from HAE36 were incubated with HA +/- SL9 peptide. Representative FACS plots show Violet dye dilution of $CD3^+CD4^+$ T cells in the absence of T_{REG} cells upon stimulation with (A) HA alone or (D) HA + SL9. Similar FACS plots are shown for Violet dye dilution of $CD3^+CD4^+$ T cells in the presence of (B) Mock T_{REG} cells + HA (E) Mock T_{REG} cells + HA + SL9 and in the presence of (C) 868^+ T_{REG} cells + HA and (F) 868^+ T_{REG} cells + HA + SL9.

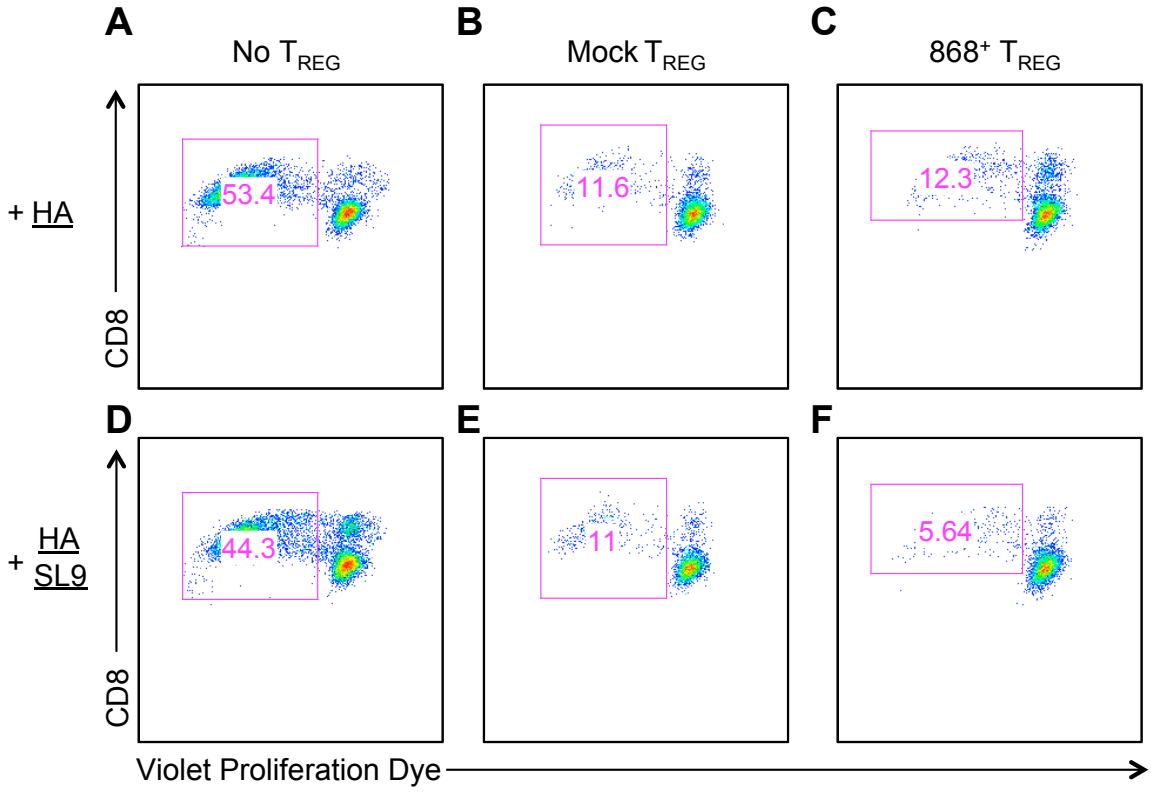

Figure 4-18 FACS plots Showing A Reduction of $CD8^+$ T cell Proliferation by Activated 868^+ T_{REG} Cells. Violet labelled PBMCs from HAE36 were incubated with HA +/- SL9 peptide. Representative FACS plots show Violet dye dilution of $CD3^+CD8^+$ T cells in the absence of T_{REG} cells upon stimulation with (A) HA alone or (D) HA + SL9. Similar FACS plots are shown for Violet dye dilution of $CD3^+CD8^+$ T cells in the presence of (B) Mock T_{REG} cells + HA (E) Mock T_{REG} cells + HA + SL9 and in the presence of (C) 868^+ T_{REG} cells + HA and (F) 868^+ T_{REG} cells + HA + SL9.

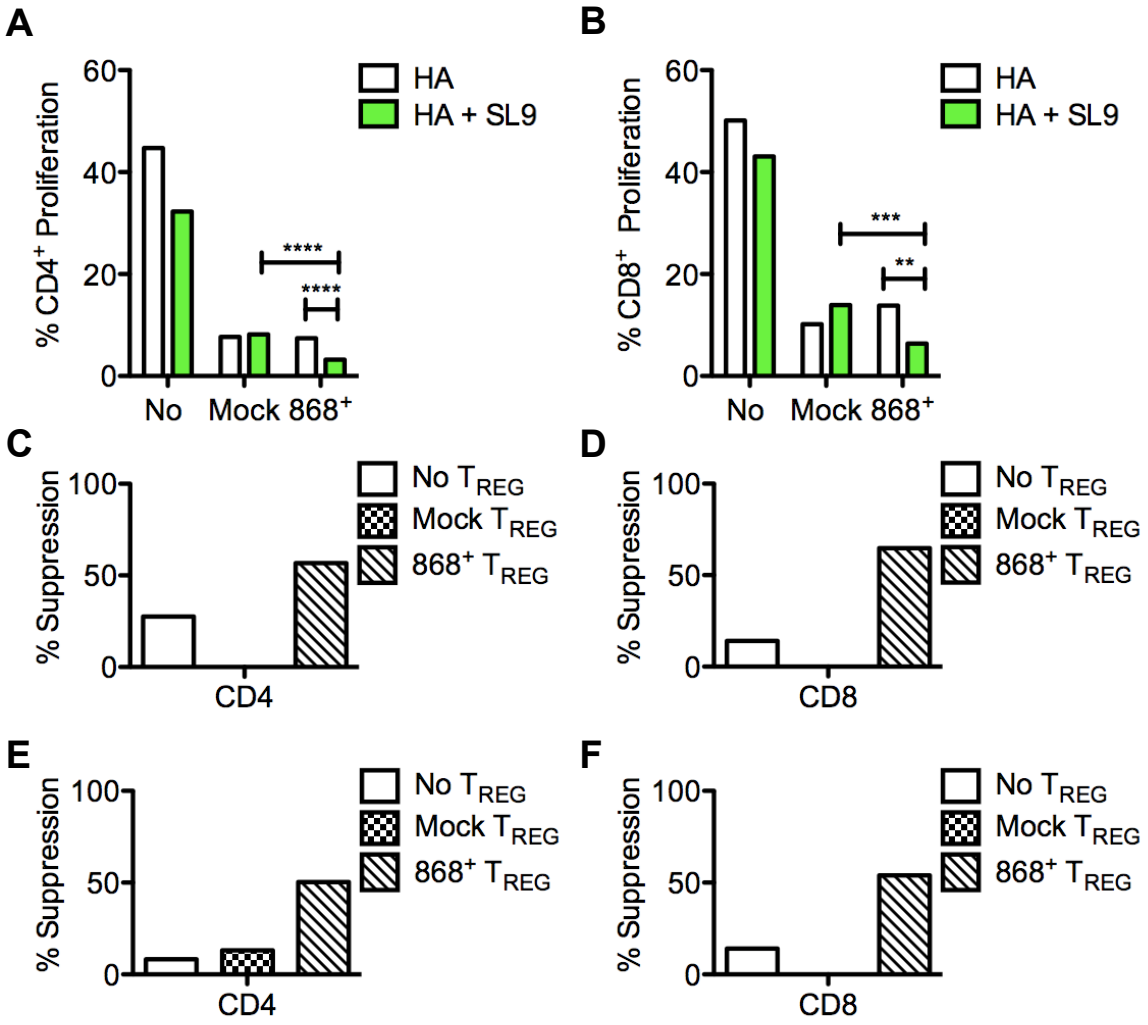

Figure 4-19 868^+ T_{REG} can Suppress The Proliferation of $CD4^+$ and $CD8^+$ T cells when Stimulated by SL9 Peptide. Bar graphs show the mean proliferation when HAE36 PBMCs are stimulated by HA +/- SL9 in the absence of T_{REG} or presence of either Mock T_{REG} or 868^+ T_{REG} for both responding (A) $CD3^+CD4^+$ and (B) $CD3^+CD4^-$ T cells. Bar graphs show the mean percentage suppression of proliferation of HAE36 PBMCs stimulated with HA Ag in the presence of SL9 peptide with either no T_{REG}, Mock Transduced T_{REG} or 868^+ T_{REG} for both (C) $CD3^+CD4^+$ and (D) $CD3^+CD8^+$ T cells. The mean percentage suppression of proliferation was calculated in an independent experiment for HAE11 upon addition of SL9 peptide for both (E) $CD3^+CD4^+$ and (F) $CD3^+CD8^+$ T cells.

4.3.8. Autoreactive MHCI TCR Transduced T_{REG} cells only Exhibit Ag Specific Suppression When Stimulated By a High Affinity Ligand

Similar suppression assays were performed using autoreactive $1E6^+$ T_{REG} cells from HAE36, to examine their ability to suppress in response to cognate ALW peptide or super agonist RQF peptide. In the absence of T_{REG} cells, a robust proliferative response by HAE36 $CD4^+$ T cells to the HA Ag was observed. This proliferative response was not significantly affected by the presence of ALW or RQF peptide (Figure 4-20 (A&D&G)). The addition of Mock or $1E6^+$ T_{REG} cells caused a marked reduction in the proliferative response of HA specific $CD4^+$ T cells, again demonstrating the presence of "non-Ag specific" suppression with both T_{REG} cell populations (Figure 4-20 (A-C)). No further reduction in $CD4^+$ T cell proliferation was observed when Mock T_{REG} cells (Figure 4-20 (B&E)) or $1E6^+$ T_{REG} cells (Figure 4-20 (C&F)) were stimulated with ALW peptide. In contrast, when $1E6^+$ T_{REG} cells were stimulated with the super agonist RQF peptide, the proliferation of HA specific $CD4^+$ T cells was further reduced (Figure 4-20 (C&I)). However, no such reduction was seen when Mock T_{REG} cells were stimulated with RQF peptide (Figure 4-20 (B&H)). A similar pattern of proliferation was observed for HAE36 $CD8^+$ T cell responses, with addition of both Mock and $1E6^+$ T_{REG} cells leading to a significant reduction in HA specific proliferation in the absence of ALW or RQF peptide (Figure 4-21 (A-C)). Similarly, no further reduction in $CD8^+$ T cell proliferation was observed upon stimulation of Mock T_{REG} cells (Figure 4-21 (B&E)) or $1E6^+$ T_{REG} cells (Figure 4-21 (C&F)) with WT ALW peptide. However, a further reduction in $CD8^+$T cell proliferation was observed when $1E6^+$ T_{REG} cells (Figure 4-21 (C&I)), but not Mock T_{REG} cells (Figure 4-21 (B&G)), were stimulated with RQF peptide. These results demonstrate that in this donor, HAE36, $1E6^+$ T_{REG} cells were capable of mediating antigen specific suppression when stimulated with super agonist RQF peptide, but not WT ALW peptide.

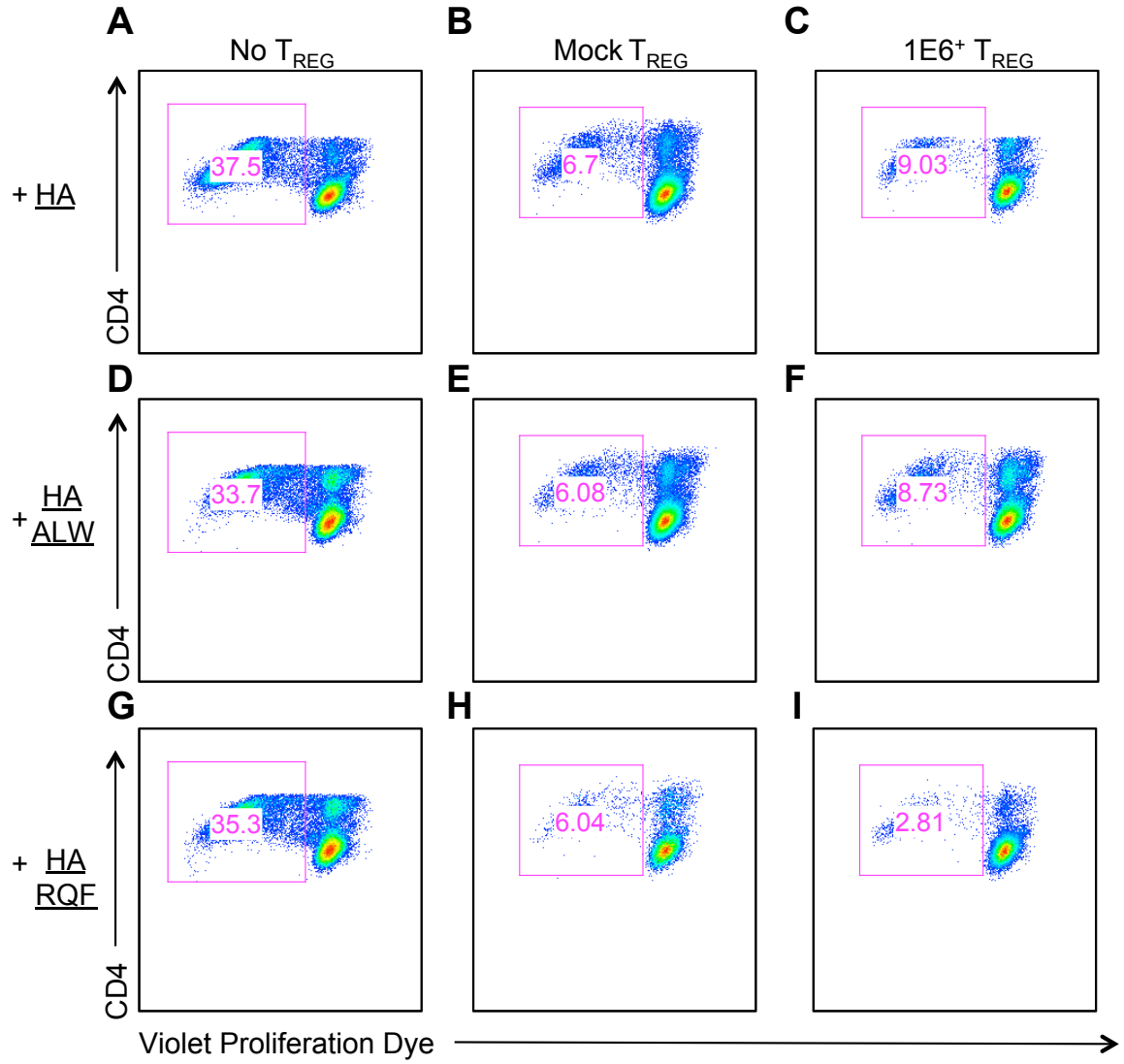

Figure 4-20 FACS plots Showing Reduction of CD4$^+$ T cell Proliferation by Super Agonist Stimulated 1E6$^+$ T_{REG} Cells. Violet labelled HAE36 PBMCs were incubated with HA +/- ALW peptide or +/- RQF peptide. Representative FACS plots show violet dye dilution of CD3$^+$CD4$^+$ T cells in the presence of HA and (A) No T_{REG} (B) Mock Transduced T_{REG} and (C) 1E6$^+$ T_{REG} cells. Similar FACS plots are shown for Violet dye dilution of CD3$^+$CD4$^+$ T cells in the presence of HA + ALW with (D) No T_{REG} (E) Mock Transduced T_{REG} and (F) 1E6$^+$ T_{REG} cells and in the presence of HA + RQF with (G) No T_{REG} (H) Mock Transduced T_{REG} and (I) 1E6$^+$ T_{REG} cells.

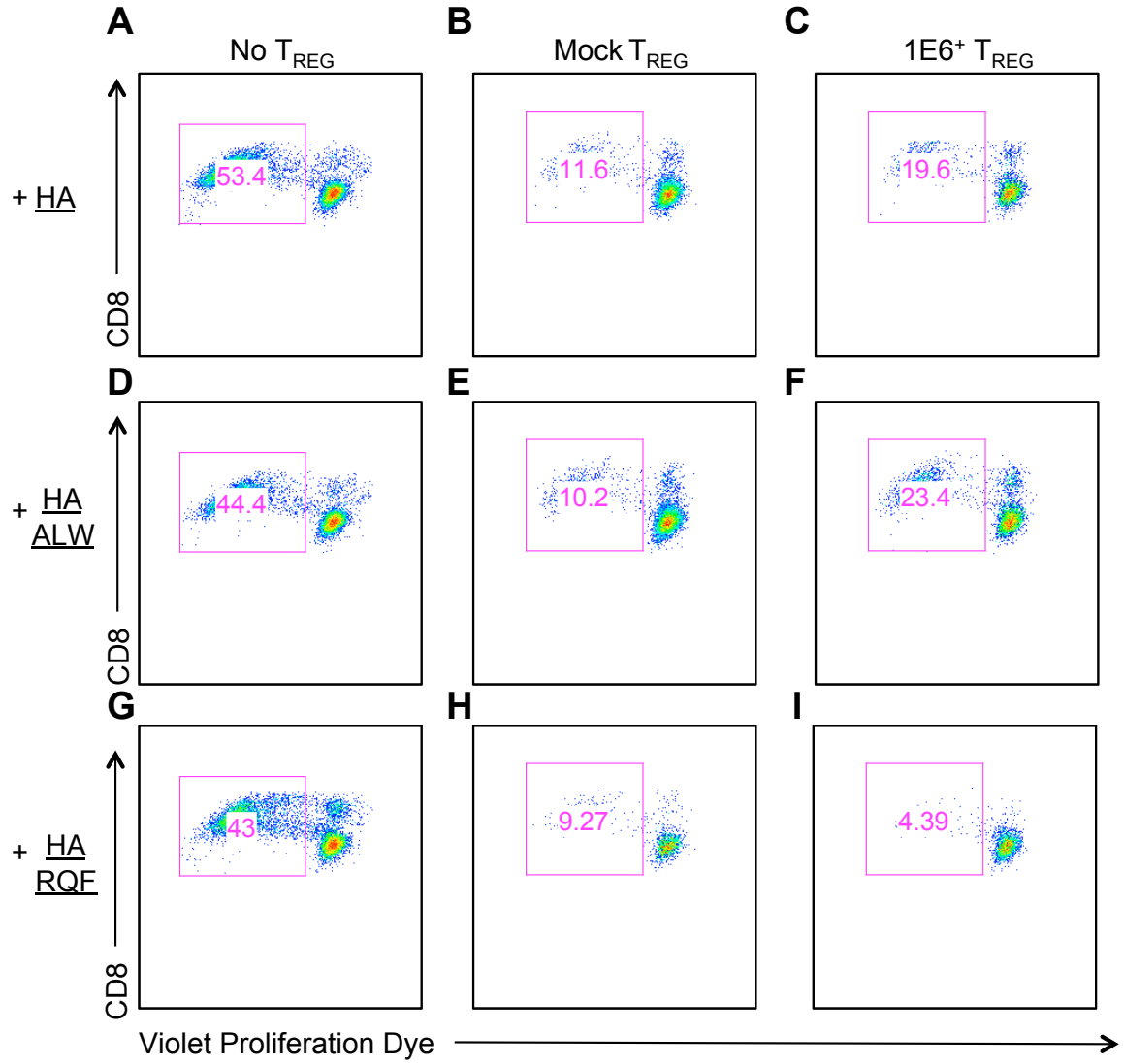

Figure 4-21 FACS plots Showing Reduction of CD8$^+$ T cell Proliferation by Activated 1E6$^+$ T_{REG} Cells. Violet labelled HAE36 PBMCs were incubated with HA +/- ALW peptide or +/- RQF peptide. Representative FACS plots show violet dye dilution of CD3$^+$CD8$^+$ T cells in the presence of HA and (A) No T_{REG} (B) Mock T_{REG} and (C) 1E6$^+$ T_{REG} cells. In the presence of HA + ALW with (D) No T_{REG} (E) Mock T_{REG} and (F) 1E6$^+$ T_{REG} cells and in the presence of HA + RQF with (G) No T_{REG} (H) Mock T_{REG} and (I) 1E6$^+$ T_{REG} cells.

Combined analysis of three independent triplicates for each culture condition are shown in Figure 4-22 (A&B) and demonstrate a reproducible and significant reduction in HAE36 CD4$^+$ and CD8$^+$ proliferation when 1E6$^+$ T_{REG} cells were stimulated with RQF, but not ALW, peptide. Ag specific suppression was quantified by calculating the percent reduction in proliferation upon the addition of ALW or

RQF peptide in the presence or absence of a particular T_{REG} cell population (Figure 4-22 (C&D)). These experiments were repeated in a second completely independent experiment using transduced T_{REG} cells and autologous PBMC from HAE11. However, in this donor, RQF stimulated $1E6^+$ T_{REG} cells could mediate Ag specific suppression of HA responding $CD8^+$ but not $CD4^+$ T cells (Figure 4-22 (E&F)), suggesting inter donor variation.

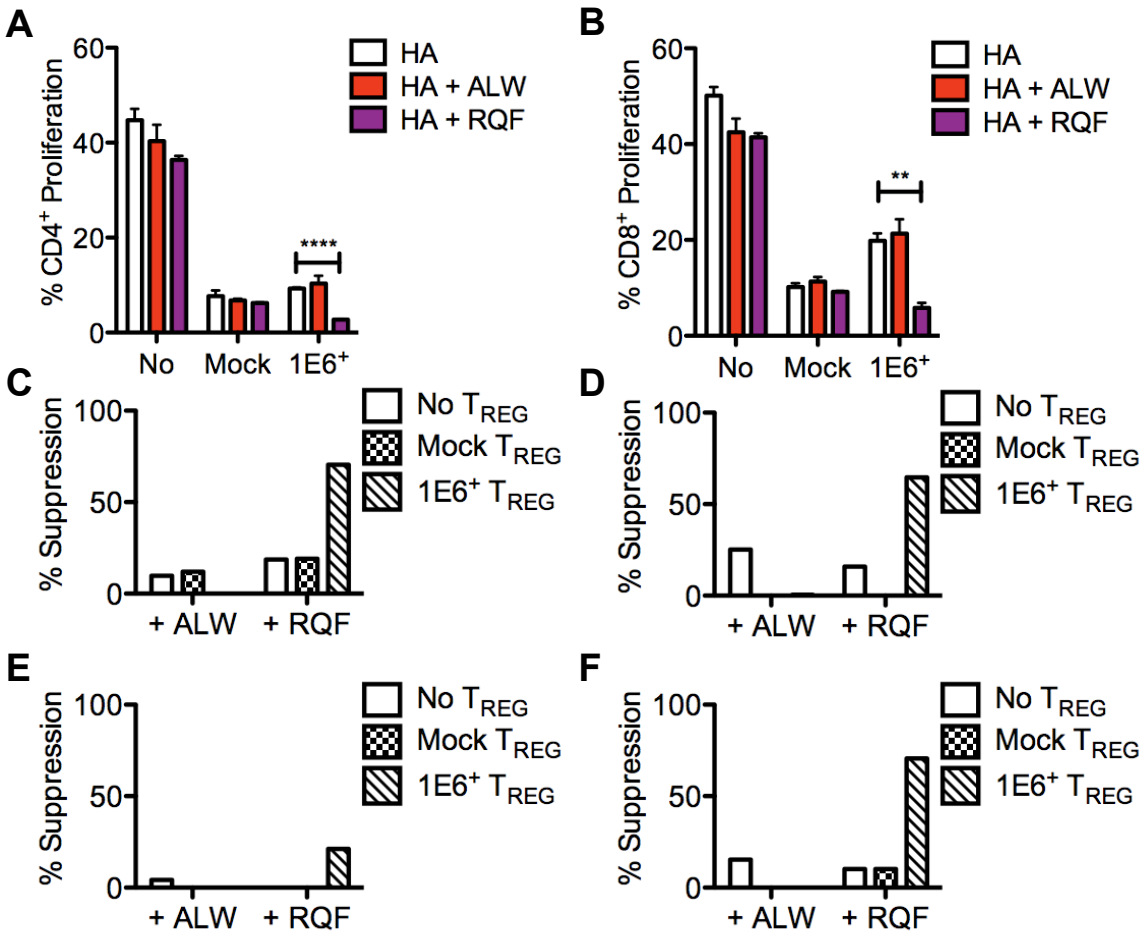

Figure 4-22 $1E6^+$ T_{REG} can Suppress The Proliferation of $CD4^+$ and $CD8^+$ T cells but only When Stimulated by A Super Agonist. Bar graphs show the mean proliferation when HAE36 PBMCs are stimulated by HA +/- ALW or +/- RQF in the presence of No T_{REG}, Mock T_{REG} or $1E6^+$ T_{REG} for responding (A) $CD3^+CD4^+$ and (B) $CD3^+CD8^+$ T cells. Bar graphs show the percent suppression of proliferation of cells from HAE36 when ALW or RQF are added to the culture in the presence or absence of T_{REG} cells (C) $CD3^+CD4^+$ and (D) $CD3^+CD8^+$ T cells. The mean percent suppression of proliferation in the presence of ALW or RQF was calculated for cells from HAE11 for (E) $CD3^+CD4^+$ and (F) $CD3^+CD8^+$ T cells.

Similar suppression assays were performed using expanded 1D7$^+$ T_{REG} cells from HAE11. In the absence of T_{REG} cells, a robust proliferative response by HAE11 CD4$^+$ T cells to the HA Ag was observed. This proliferative response was not affected by the presence of VMN peptide (Figure 4-23 (A&D)). Similarly, "non-Ag specific" suppression was observed upon addition of Mock T_{REG} and 1D7 T_{REG} cells. However, no Ag specific suppression of CD4$^+$ T cells was exhibited by 1D7$^+$ T_{REG} cells upon addition of the VMN peptide to cultures (Figure 4-23 (C&F)). A similar pattern was observed for the proliferation of HA specific CD8$^+$ T cells. Although 1D7$^+$ T_{REG} cells suppressed the proliferation of CD8$^+$ T cells via "non Ag specific" suppression, a further reduction in proliferation was not observed upon addition of VMN peptide (Figure 4-24 (C&F)).

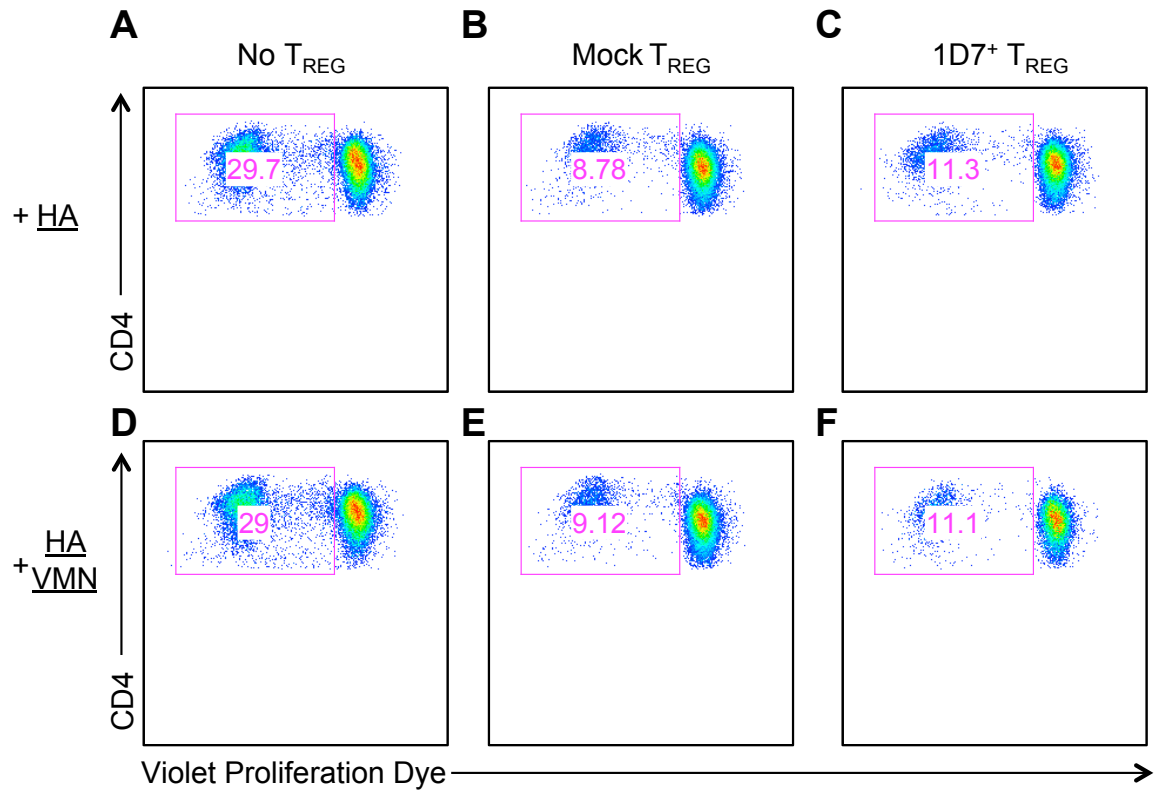

Figure 4-23 FACS plots Showing No Effect on CD4$^+$ T cell Proliferation by 1D7$^+$ T_{REG} Cells. Violet labelled PBMCs were incubated with HA +/- VMN peptide. Representative FACS plots show violet dye dilution of HAE11 CD3$^+$CD4$^+$ T cells in the presence of HA with (A) No T_{REG} (B) Mock Transduced T_{REG} and (C) 1D7$^+$ T_{REG} cells. FACS plots show violet dye dilution of CD3$^+$CD4$^+$ T cells in the presence of HA and VMN peptide with (D) No T_{REG} (E) Mock Transduced T_{REG} and (F) 1D7$^+$ T_{REG} cells.

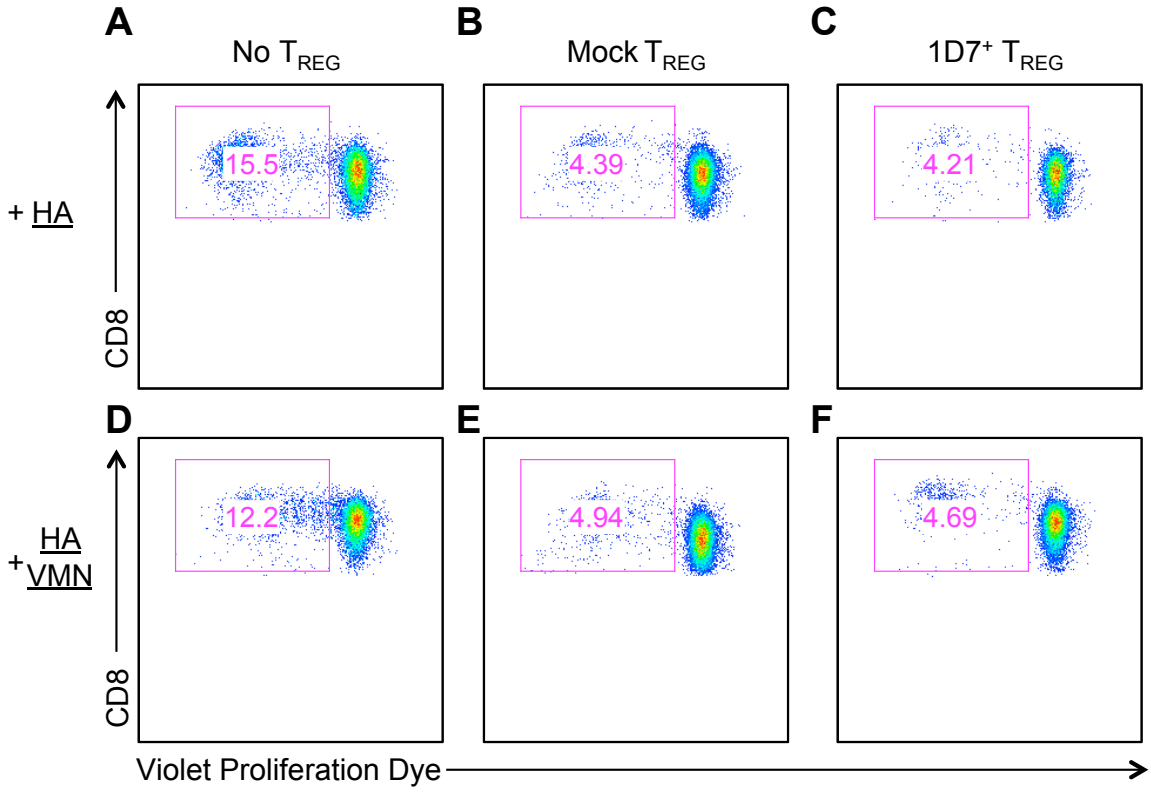

Figure 4-24 FACS plots Showing No Effect on $CD8^+$ T cell Proliferation by $1D7^+$ T_{REG} Cells. Violet labelled PBMCs were incubated with HA +/- VMN peptide in the absence of presence of indicated T_{REG} cell populations. Representative FACS plots show violet dye dilution of HAE11 $CD3^+CD8^+$ T cells in the presence of HA with (A) No T_{REG} (B) Mock Transduced T_{REG} and (C) $1D7^+$ T_{REG} cells. FACS plots show violet dye dilution of $CD3^+CD8^+$ T cells in the presence of HA and VMN peptide with (D) No T_{REG} (E) Mock Transduced T_{REG} and (F) $1D7^+$ T_{REG} cells.

Combined analysis of three independent triplicates for each culture condition are shown in Figure 4-25 (A&B) and demonstrate no reduction in the proliferation of HAE11 $CD4^+$ or $CD8^+$ T cells when $1D7^+$ T_{REG} cells when stimulated with the VMN peptide. These data demonstrate that the $1D7^+$ T_{REG} cells are not capable of mediating Ag specific suppression when stimulated by the cognate VMN peptide.

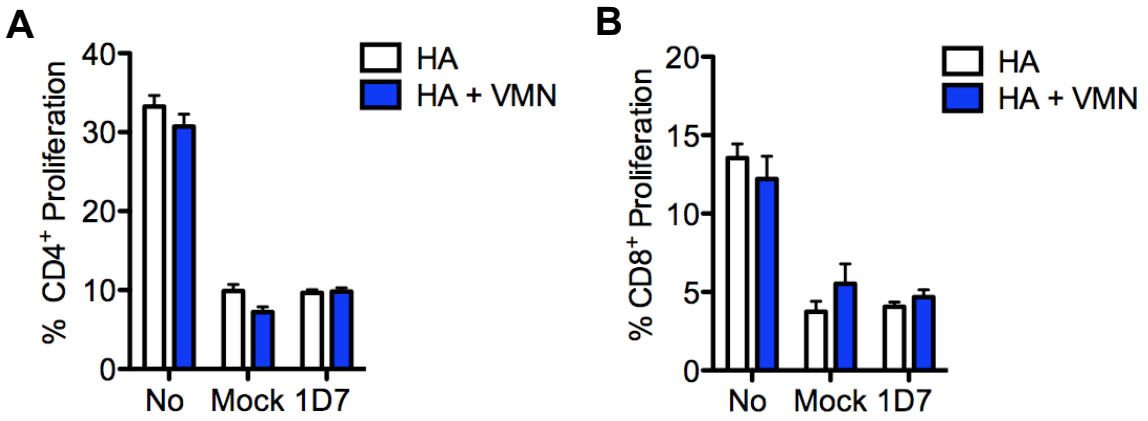

Figure 4-25 1D7$^+$ T_{REG} cells Can Not Suppress Proliferation of T Cells In the Presence of VMN Peptide. Bar graphs show the mean proliferation when HAE11 PBMCs are stimulated by HA +/- VMN in the presence of No T_{REG}, Mock Transduced T_{REG} or 1D7$^+$ T_{REG} for both responding (A) CD3$^+$CD4$^+$ and (B) CD3$^+$CD8$^+$ T cells

4.3.9. Expansion of Transduced T_{REG} Cells Using Rapamycin.

During the course of these studies it was noted that expansion of T_{REG} cells under the conditions described above resulted in varying levels of T_{REG} cell purity. In agreement with other members of the laboratory performing T_{REG} cell expansions, it was determined that loss of FoxP3 expression and outgrowth of T_{EFF} cells that occurred within T_{REG} cultures was a particular problem when Tregs were subjected to >2 cycles of expansion and was highly variable between donors. Other studies have identified a similar loss of FoxP3 expression during long term *in vitro* polyclonal stimulation of CD4$^+$CD25$^+$CD127^{lo} T_{REG} cells (Hoffmann et al., 2009; Hoffmann et al., 2006). It has been suggested, that long term *in vitro* culture can result in both epigenetic changes at the *FoxP3* locus and loss of FoxP3 expression. This loss of FoxP3 expression can also correlate with loss of T_{REG} cell suppressive function (Hoffmann et al., 2009). As suppression of CD4$^+$ T cells by RQF stimulated 1E6$^+$ T_{REG} cells was observed in only 1 of 2 donors, the Ag specific capabilities of these cells required further examination. Methods were therefore sought to improve the phenotype of expanded T_{REG} cell populations. Rapamycin

(RAPA) is an immunosuppressive agent that inhibits the mechanistic Target Of Rapamycin (mTOR) pathway and therefore blocks cell responsiveness to the IL-2 pathway. It has been shown that this block in IL-2 responsiveness is selective for effector $CD4^+$ and $CD8^+$ T cells and consequently, $CD4^+$ T_{REG} cells can selectively expand in RAPA treated cultures of T cells (Battaglia et al., 2006). Additionally, RAPA has been shown to selectively outgrow the naïve and stable $CD4^+CD25^+CD45RA^+$ T_{REG} compartment, which has been identified as having superior suppressive capabilities compared to $CD4^+CD25^+CD45RA^-$ T_{REG} cells (Hoffmann et al., 2006; Miyara et al., 2009; Scotta et al., 2013). Therefore, using T_{REG} cells from a third healthy control donor, T01, RAPA was added to the culture of transduced T_{REG} cells to determine whether it could improve the phenotype and suppressive function of the transduced T_{REG} cells. Isolation of T_{REG} cells by FACS from this particular donor led to a low recovery of T_{REG} cells of only $4x10^5$. Therefore, isolated T_{REG} cells were split into two cultures on Day 2 and only transduced with the 1E6 or 868 TCR, and not with the Mock vector. RAPA has been shown to reduce the kinetics of T_{REG} cell growth in mice, and indeed the protocol for the expansion of T_{REG} cells had to be adapted from 8 day cycles of expansion to 12 days (Figure 4-26).

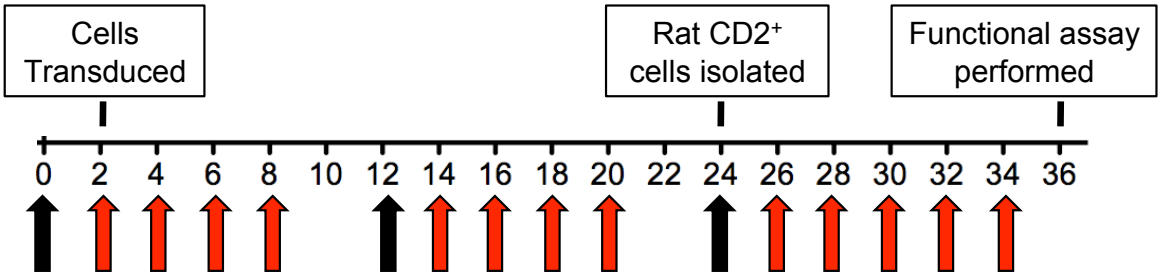

Figure 4-26 Protocol for Expansion of $CD4^+$ T_{REG} with RAPA. $CD4^+$ T_{REG} were cultured in the presence of 600IU/ml IL-2 and 100ng/ml RAPA. Black arrows indicate when the cells were stimulated with αCD3/CD28 beads and red arrows indicate when cells were given fresh media with IL-2 and RAPA.

Despite this reduction in kinetics of T_{REG} cell growth, transduction of T_{REG} cells cultured in RAPA with the 1E6 and 868 TCR containing LV was not altered. The

percentage of cells transduced with the 1E6 LV was 25% and 42% with the 868 LV (Mean % transduction without RAPA 25.98 +/- 11.6% SD and 32.6 +/- 10% SD for 1E6 and 868 respectively) (data not shown). The phenotype of cells expanded in RAPA after each of the three rounds of expansion, D12, D24 and D36 (Figure 4-27) was tested. At all time-points examined, >90% of expanded T_{REG} cells were $CD3^+CD4^+$ (Figure 4-27 (A)). The FoxP3 expression of expanded T_{REG} cells was also measured and a minimal loss of FoxP3 expression observed between D12 and D24 of culture. However, an increased loss of FoxP3 expression was observed between D24 and D36 of culture, with FoxP3 expression reducing from 87.9% to 67.7% (Figure 4-27 (B)).

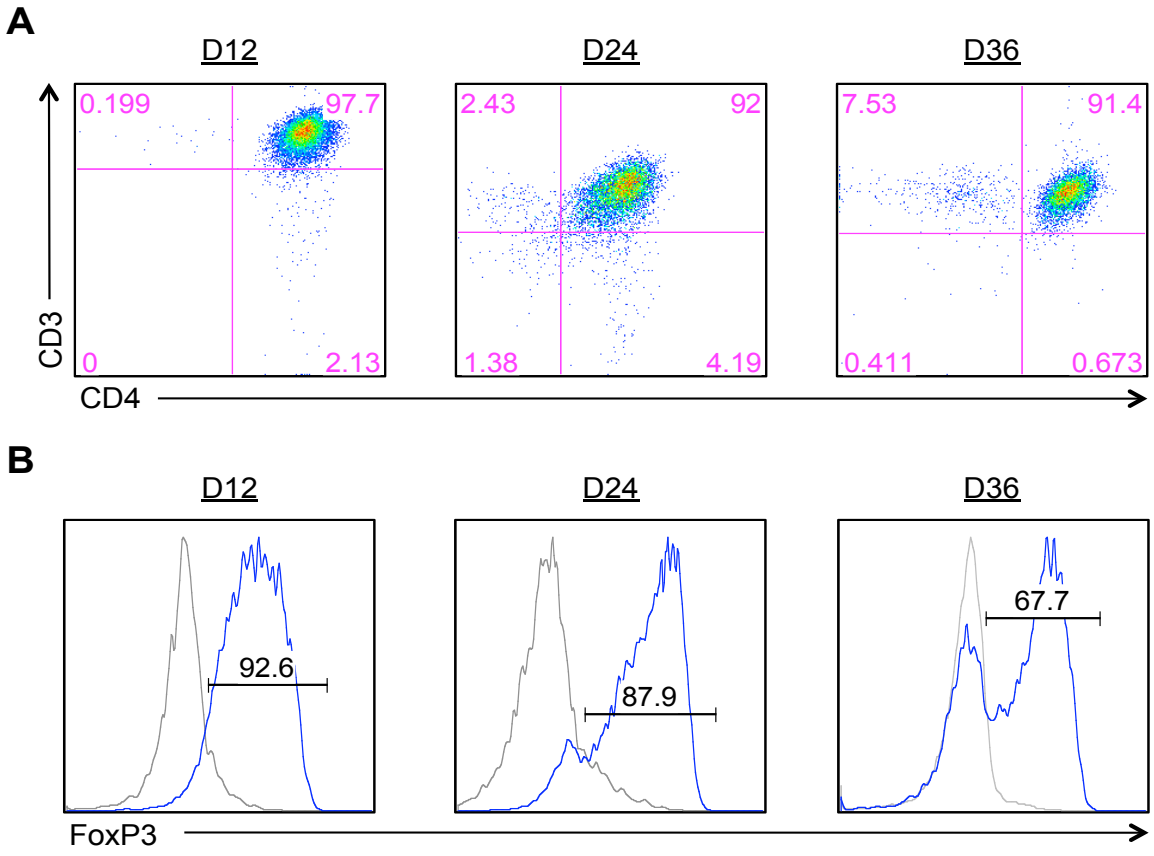

Figure 4-27 Phenotype of Transduced $CD4^+$ T_{REG} Expanded Using RAPA. The phenotype of T01 transduced $CD4^+$ T_{REG} cells was assessed at the end of each round of expansion on D12, D24 and D36. Cells were surface stained with (A) αCD3 and αCD4 antibodies and (B) stained intracelullar for FoxP3 expression. Grey line indicates FoxP3 expression on transduced $CD4^+$ T_{EFF} cells. FACS plots are representative of expansion of both 1E6 and 868 transduced T_{REG} cells.

At the end of the third round of expansion, the expanded Rat $CD2^+$ sorted T_{REG} cells were cryopreserved and then thawed for use in an Ag specific suppression assay. For this assay, as Mock transduced T_{REG} cells were unavailable, 868^+ T_{REG} cell served as a control T_{REG} population for suppression assays with $1E6^+$ T_{REG} cells. Similar to the assays shown previously, $CD4^+$ and $CD8^+$ T cells exhibited a robust proliferative response to HA Ag, which was not significantly altered by the addition of ALW or RQF peptide (Figure 4-28 (A-B)). The HA proliferative response of both $CD4^+$ and $CD8^+$ T cells was reduced to a similar extent in the presence of $1E6^+$ T_{REG} cells and 868^+ T_{REG} cells again demonstrating non Ag specific suppression. The addition of RQF peptide, but not ALW peptide, caused a further significant reduction in proliferation of $CD4^+$ and $CD8^+$ T cells by $1E6^+$ T_{REG} cells. The addition of these two peptides did not alter the suppression exhibited by 868^+ T_{REG} cells (Figure 4-28 (A-B)). These data again demonstrated Ag specific suppression by $1E6^+$ T_{REG} cells when stimulated with RQF peptide. The percentage suppression induced by stimulation of each cell type by different peptides was calculated. The percentage suppression of proliferating $CD4^+$ T cells upon addition of $1E6^+$ T_{REG} cells and RQF peptide was 82.7%. No suppression was observed upon addition of $1E6^+$ T_{REG} cells and ALW peptide (Figure 4-28 (C)). Similarly, the percentage suppression of proliferating $CD8^+$ T cells was 87.1% upon addition of $1E6^+$ T_{REG} cells and RQF peptide (Figure 4-28 (D)).

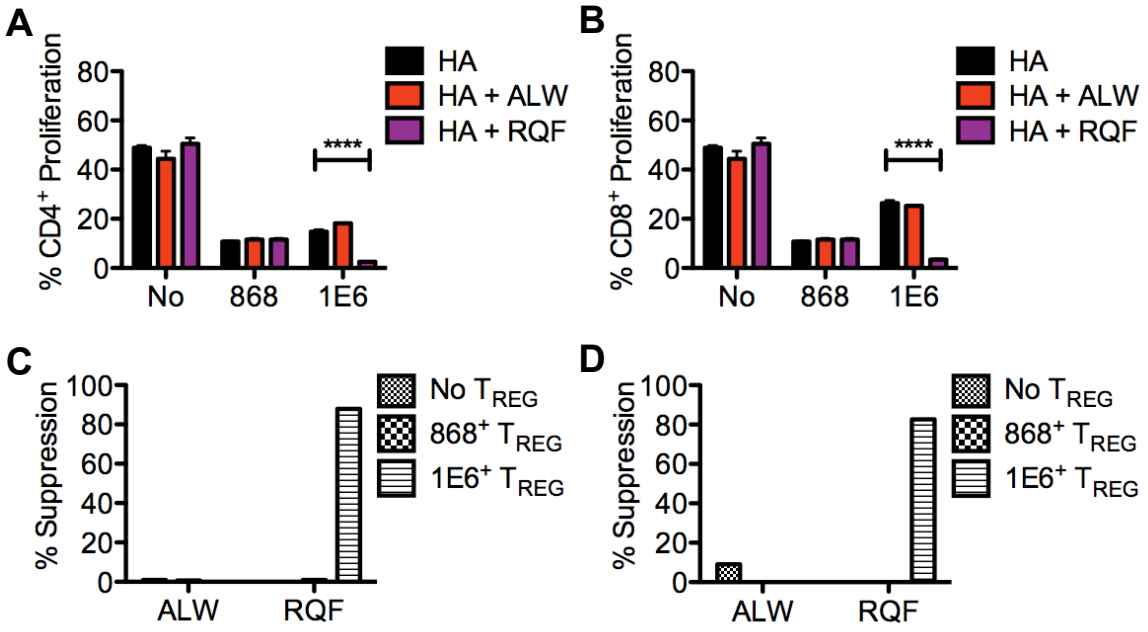

Figure 4-28 Ag Specific Suppression by 1E6$^+$ T_{REG} Cells Expanded in RAPA. Bar graphs show the mean proliferation when T01 PBMCs were stimulated by HA +/- ALW or +/- RQF in the presence of No T_{REG}, 868$^+$ T_{REG} or 1E6$^+$ T_{REG} for both responding (A) CD4$^+$ and (B) CD8$^+$ T cells. Bar graphs show the percent suppression of proliferation of T01 cells when either ALW or RQF is present in the culture with no T_{REG}, 868$^+$ T_{REG} or 1E6$^+$ T_{REG} for both (C) CD4$^+$ and (D) CD8$^+$ T cells.

Taken together these studies confirm that when stimulated with RQF, 1E6$^+$ T_{REG} cells can exhibit Ag specific suppression of CD8$^+$ T cells, and although not seen in every donor, CD4$^+$ T cell responses. However in all cases, a large degree of "non Ag specific" suppression was also observed with all T_{REG} cell populations at a ratio of 5 PBMCs: 1 T_{REG} cell. We therefore sought to investigate the effect of different ratios of 1E6$^+$$T_{REG}$ cells on the reduction in proliferation of HA responding CD4$^+$ and CD8$^+$ T cells. In this assay the "non Ag specific" suppression of proliferating CD4$^+$ and CD8$^+$ T cells could be determined. This was the suppression of proliferation observed when 1E6$^+$ T_{REG} cells were added to the culture without the addition of RQF peptide. This could therefore be compared to the Ag specific suppression exhibited by 1E6$^+$ T_{REG} cells stimulated by the super agonist RQF peptide. The ratios of PBMCs to T_{REG} cells investigated were 5:1, 10:1, 20:1 and 40:1. All percentage suppression calculations were carried out compared to the

absence of T_{REG} cells. At all ratios tested the $1E6^+$ T_{REG} cells exhibited greater suppression of $CD4^+$ T cell proliferation in the presence of RQF peptide (Range 98.1 - 50.1%) compared to the absence of RQF peptide (Range 69.7 – 6.6%) (Figure 4-29 (A)). For comparison of this suppression, the ratio of PBMCs to T_{REG} cells required to achieve 50% suppression of proliferating $CD4^+$ and $CD8^+$ T cells was estimated. To achieve 50% suppression of $CD4^+$ T cell proliferation in the absence of RQF peptide, a ratio of PBMC:T_{REG} cells of ~ 8:1 was required. To achieve the same percentage suppression in the presence of RQF peptide a much lower PBMC:T_{REG} cell ratio of 40:1 was required (Figure 4-29 (A)). Similarly, at all ratios tested the $1E6^+$ T_{REG} cells exhibited greater suppression of proliferating $CD8^+$ T cells in the presence of RQF peptide (Range 94.8 – 36.7%) than in the absence of RQF peptide (Range 58.16 – 6.97%) (Figure 4-29 (B)). For 50% suppression of HA responding $CD8^+$ T cells an estimated ratio of 6:1 PBMC:T_{REG} cells would be required in the absence of RQF peptide. However in the presence of RQF peptide a lower PBMC:T_{REG} cell ratio of 20:1 would be required. Thus, the potency of suppression of both $CD4^+$ and $CD8^+$ T cells exhibited by $1E6^+$ T_{REG} cells is roughly 3-5 fold higher when the T_{REG} cells are stimulated via the introduced TCR.

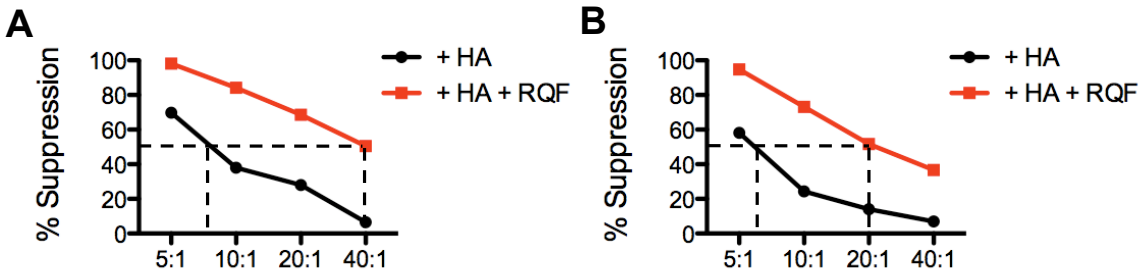

Figure 4-29 Ag Specific T_{REG} cells expanded in RAPA can suppress proliferation at low T_{REG}:PBMC ratios. Violet labelled T01 PBMCs were stimulated with HA Ag either in the presence or absence of RQF peptide. Suppression under both conditions when $1E6^+$ T_{REG} were added to the culture was calculated by ((%proliferation No T_{REG} / % Proliferation $1E6^+$ T_{REG}) / %proliferation No T_{REG})*100 for proliferation of responding (A) $CD4^+$ and (B) $CD8^+$ T Cells. The dotted line represents the ratio of $1E6^+$ T_{REG} cells required for 50% suppression when they are un-stimulated in culture or stimulated with RQF peptide.

4.4. Discussion

These experiments were designed to test the hypothesis that although autoreactive MHCI Restricted TCRs cannot re-direct the Ag specificity of a $CD4^+$ T_{EFF} cell, they can re-direct the Ag specificity of a $CD4^+$ T_{REG} cell (Plesa et al., 2012). Similar to the study carried out in Jurkat cells, a step-wise approach was taken to investigate the expression of the three MHCI restricted TCRs, their ability to signal in primary T cell populations and most importantly their ability to confer Ag specific suppressive capabilities to a $CD4^+$ T_{REG} cell.

Firstly, it was important to test that all primary T cell populations examined could be successfully transduced with LV under the current protocols of activation. This study demonstrates, as others have shown that, $CD4^+$ T_{EFF}, $CD8^+$ T cells and $CD4^+$ T_{REG} cells can be transduced with LV (Brusko et al., 2010; Dardalhon et al., 2001). It was surprising to note, that under the protocols followed, $CD4^+$ T_{REG} were significantly more susceptible to transduction with TCR containing LV compared to $CD4^+$ T_{EFF} and $CD8^+$ T cells. Other have shown superior transduction of both $CD4^+$ T_{EFF} and $CD8^+$ T cells (Dardalhon et al., 2001), however as the main focus of this project was $CD4^+$ T_{REG}, further optimisation to increase transduction of these cell types was not explored. It was also important to determine that the transduced T_{REG} cells could be expanded to gain significant numbers of these cells for experiments. After each round of expansion, a 10-20-fold increase in T_{REG} cell numbers was observed. Over three round of expansion, this resulted in ~1000 fold expansion, which is consistent with what other studies have observed using polyclonal expansion of non-transduced T_{REG} cells (Putnam et al., 2009). Unfortunately, as $CD4^+$ T_{REG} cells from each donor were typically split four ways to be transduced with Mock, 868, 1E6 and 1D7 LV the number of $CD4^+$ T_{REG} cells was limited and therefore a side by side comparison of the expansion rates of non transduced and transduced cells was not possible. In general, transduced and expanded $CD4^+$ T_{REG} cells remained relatively stable during the expansion process,

with >75% maintenance of $CD3^+CD4^+FoxP3^+$ cells at the end of the 24 day expansion period.

The observation that autoreactive MHCI TCRs have a different pattern of expression to the 868 pathogen specific TCR in transduced primary T cell populations was similar to previous observations in Jurkat cell lines in chapter 3. The 868 pathogen specific TCR was readily expressed on the cell surface of transduced primary T cell populations as measured by consistent dual expression of the clonotypic vβ5 chain and Rat CD2. In contrast to this, when primary T cell populations were transduced with either the autoreactive 1E6 or 1D7 TCRs, only a proportion of the transduced Rat $CD2^+$ cells co-expressed the clonotypic vβ chain of the respective TCRs. There are at least two explanations for this phenomenon, one is that the autoreactive TCRs are unable to compete with the endogenous TCR for CD3 assembly and the second is that the α and β chains of the autoreactive TCRs do not preferentially pair with one another. Turning first to the ability of TCRs to compete for assembly with CD3 chains, others have shown that TCRs can be classed as "strong" or "weak" TCRs based on their ability to complex with CD3 molecules (Stauss et al., 2007). It was demonstrated by Heemskerk and colleagues that expression levels of a retroviral introduced TCR depended on which endogenous TCR was expressed by the transduced cell (Heemskerk et al., 2007). This variation in introduced TCR expression was due to the preferential binding affinity of the endogenous TCR for the CD3 complex over the introduced TCR. Due to the limited number of CD3 complexes within a cell, TCRαβ pairings that have a higher binding affinity for CD3 will be preferentially expressed on the cell's surface in situations where two TCRs are present in a cell. The group of Hans Stauss, who has proffered the terms "weak" and "strong" TCRs, demonstrated that CD3 complexes are rate-limiting. In one study they showed that by increasing the availability of CD3 complexes using retroviral transduction of a CD3 expressing vector, they could increase the expression of a 'weak" introduced TCR by up to 26 fold (Ahmadi et al., 2011). Therefore, by increasing the amount of available CD3 complexes the differences in expression of "weak" vs. "strong" TCRs

are less apparent. The ability of the 1E6 and 1D7 TCRs to be expressed may also be due to the inefficient pairing of the TCRα and TCRβ chains of these TCRs. In chapter 3, this study showed that in Jurkat cells, which possessed no endogenous TCR, the autoreactive TCRs were poorly expressed compared to the pathogen specific 868 TCRs. This study also showed that in primary T cells populations, there was a trend for a reduced level of introduced autoreactive TCR clonotypic vβ expression compared to the endogenous vβ expression as measured by MFI. Although these trends were not significant, no such trend was observed for the 868 TCR vβ5, which was comparable to endogenous vβ5 expression. Therefore it can be suggested that the reduced ability of the 1E6 and 1D7 TCRs to be expressed on the surface of a T cell may be due both to inefficient pairing and a reduced affinity for CD3. In fact, Heemskerk and colleagues have suggested that the ability to complex with CD3 may be reliant on the interchain affinity of a TCR's α and β chain (Heemskerk et al., 2007). Thus, it is possible to class the pathogen specific 868 TCR as a "strong" TCR that has high interchain affinity and therefore a strong affinity for CD3 complexes allowing it to outcompete the majority of endogenous TCRs for cell surface expression. However, both the autoreactive 1E6 and 1D7 appear to be "weak" TCRs, whose low TCRα and TCRβ interchain affinity culminate in a reduced ability to assemble in a TCR-CD3 complex. Whether this is true of all pathogen specific and autoreactive TCRs would require a larger panel of TCRs with both reactivity's and was beyond the scope of this study.

The signalling capabilities of each TCR in the three primary T populations transduced was very similar to the results observed in model T cell lines. As expected, $CD4^+$ T_{REG}, T_{EFF} and $CD8^+$ T cells transduced with the 868 TCR were capable of up-regulating CD69 in response to the SL9 peptide. However, in keeping with previous data, $CD4^+$ T_{EFF} or T_{REG} cells transduced with the 1E6 TCR could not respond to its preproinsulin ALW peptide by up-regulating CD69. Most surprisingly, $CD8^+$ T cells transduced with the 1E6 TCR also failed to up-regulate CD69 in response to the ALW peptide. This was particularly surprising when this observation is linked to the fact that expression of CD8α alone was able to rescue

the signalling of the 1E6 TCR in a Jurkat cell. It is possible however that due to the weak expression of the 1E6 TCR on the surface of the transduced T cells that the overall avidity of the 1E6 TCR ALW-MHCI interaction is insufficient for effective signalling despite the presence of the CD8 co-receptor. The productive hit rate model of TCR activation indicates that serial triggering of TCRs is required for full TCR signalling i.e. multiple TCRs must bind to pMHC before effective signalling can occur (Valitutti et al., 1995). Additionally it has been found that there is an optimal dwell time (length of interaction between pMHC and TCR) for effective TCR signalling, and a dwell time shorter or longer than this time can impair TCR signalling (Kalergis et al., 2001). The structural characteristics of the 1E6 TCR and its affinity for the index peptide ALW was recently described in a study published in 2012 (Bulek et al., 2012). Significantly, in this study they showed that the kinetics of the interaction between the 1E6 TCR and the ALW peptide were too rapid too measure by SPR kinetic titration analysis, which would therefore lead to an overall short, and likely insufficient dwell time for TCR activation. In combination with the low expression of the 1E6 TCR on the surface of the transduced T cells, these factors could lead to an overall non productive TCR signal in response to the ALW peptide.

Another noteworthy observation with regards to the 1E6 TCR was the ability of cells transduced with this TCR to up-regulate CD69 in response to the super-agonist RQF peptide. This showed that this TCR could signal in any of the T cells transduced, when the TCR affinity for pMHC reached the threshold required for activation. Furthermore, the up-regulation of CD69 was not exclusive to the Rat $CD2^+$ cells that co-expressed TCRvβ8 – but could also be seen in cells that were Rat $CD2^+$ and TCRv$\beta8^-$ (importantly not in cells that were TCRv$\beta8^+$Rat $CD2^-$). It is possible that cells that appear TCRv$\beta8^-$ are actually TCRv$\beta8^{low}$ and that the high affinity interaction of the RQF peptide and TCR can overcome the poor TCR expression by increasing the amount of TCRs that are triggered with an optimal dwell time. A second possibility is that cells taken to be 1E6 TCR^- based on their lack of expression vβ8 expression could in fact be expressing the vα chain of the

1E6 TCR, which potentially has paired with the transduced cells endogenous vβ. Studies in mice from the group of George Eisenbarth showed that when a TCRα chain of an insulin autoreactive T cell clone was paired with various TCRβ chains, these TCR pairings could still promote the production of insulin reactive antibodies (Kobayashi et al., 2008). However, the mice baring only the TCRα chain of the insulin reactive TCR did not develop the insulitis seen in mice that were fully transgenic for the original TCRα and TCRβ chain of the insulin reactive TCR. Suggesting that although the TCRα chain could confer insulin specificity alone it was optimal when paired with the original vβ chain of this autoreactive TCR. It is therefore plausible that the vα of the 1E6 TCR could be conferring Ag specificity when paired with endogenous vβ of transduced cells, which allows response to the RQF peptide. Currently, as there is no vα chain antibody available for the 1E6 TCRvα12-3.1 chain this hypothesis would be difficult to test. Potentially, a LV vector that solely encoded the vα12-3.1 chain could be used to evaluate this, by testing the response of vα12-3.1 transduced cells to the RQF peptide. Similarly to the 1E6 TCR, the cells transduced with the 1D7 TCR were also unable to up-regulate CD69 in response to its index peptide VMN. Unfortunately, due to a lack of a super-agonist peptide for this TCR it was impossible to determine if the 1D7 TCR behaves the same as the 1E6 TCR or if its specificity for the VMN peptide was compromised during the clonotyping of this TCR.

The study by *Plesa et al* looked at the ability of the low affinity NY-ESO-1 specific TCR to signal in a T_{REG} cell by looking at its ability to confer Ag specific suppressive capabilities. It is unclear how strong (or weak) a TCR signal is required by a T_{REG} to become suppressive vs. up-regulating CD69. In fact, studies in $CD8^+$ T cells have identified a hierarchy in effector function dependent on the level of TCR stimulation a cell receives (van den Berg et al., 2013). It was therefore pertinent to measure whether or not the MHCI TCRs could confer Ag specific capabilities, despite the failure of T_{REG} cells transduced with these TCRs to up-regulate CD69 in response to peptide. Therefore, an Ag specific suppression assay

was developed to measure whether the autoreactive MHCI TCRs could confer Ag specific capabilities to a $CD4^+$ T_{REG} cell. In this assay, autologous PBMCs were stimulated with a recall Ag, HA, in order to have naturally responding proliferating T cells to suppress. To these cultures, combinations of sorted transduced T_{REG} cells and their specific peptides were added. Therefore, the ability of transduced T_{REG} to suppress the proliferation of responding HA T cells when they were stimulated by their specific peptide compared to the absence of peptide specific stimulation could be assayed. Consistent with all other results, the 868^+ T_{REG} cells, were capable of suppressing the proliferation of HA specific $CD4^+$ and $CD8^+$ T cells in an Ag specific manner. In contrast, mock LV transduced T_{REG} cells that solely expressed Rat CD2, did not. In this assay we observed high levels of "non-Ag Specific" suppression i.e. suppression when 868^+ T_{REG} were not exposed to SL9 peptide, or when mock LV transduced T_{REG} cells were added to the culture. In this study, this observation has been termed "non-Ag specific" suppression as it occurs without antigenic stimulation of the $CD4^+$ T_{REG} cells via their introduced TCRs. However, there are a number of possible explanations for the high level or suppression exhibited by the non-peptide stimulated $CD4^+$ T_{REG} cells. It has been well characterised that $CD4^+$ T_{REG} cells require stimulation via the TCR before becoming suppressive (Thornton and Shevach, 2000) and indeed the T_{REG} cells used in these assay had been pre-activated with αCD3/CD28 beads during the course of their expansion. Therefore, it is possible that the T_{REG} cells were still activated when used in the suppression assays. However, to minimise this possibility, the expanded T_{REG} cells were rested without beads and IL-2 for 48 hours, cryopreserved and then thawed for use in the suppression assays. Another possibility is that the expanded $CD4^+$ T_{REG} cells were specific for pMHCII complexes on the surface of APC's within the culture, and after becoming activated through the endogenous TCR elicited suppressive function. A seminal study by *Taams et al* showed that $CD4^+CD25^+$ T_{REG} cells were able to exhibit suppressive function against a range of Ags, including self-Ag human heat Shock Protein 60 (hHSP60) and tetanus toxoid (Taams et al., 2002). This study also used a panel of TCRvβ antibodies to demonstrate that the TCRvβ usage of isolated $CD4^+CD25^+$ T

cells was no different to the usage of $CD4^+CD25^-$ T_{EFF} cells. Therefore, it is possible that a small number of Ag specific T_{REG} cells within the culture were specific for either self-pMHCII on the surface of APCs, or for the HA Ag. T_{REG} cells with these specificities in the culture could therefore have become activated to suppress the HA specific proliferative responses of the $CD4^+$ and $CD8^+$ T cells. Although, determining the exact nature of this "non-Ag specific" suppression would be beyond the scope of this study, it is clear from clinical data from the adoptive transfer of polyclonal T_{REG} cells that these cells are capable of suppressing T cell responses (Riley et al., 2009). However, it is also apparent from the data shown here that larger populations of $CD4^+$ T_{REG} cells activated in an Ag specific manner were more efficacious at eliciting suppressive function.

In contrast to the study by *Plesa et al*, this study showed that low affinity MHCI restricted TCRs were unable to re-direct the Ag specificity of $CD4^+$ T_{REG} cells. In an Ag specific suppression assay, $1E6^+$ T_{REG} cells were unable to exhibit suppressive function when stimulated by the WT ALW peptide. However, when these cells were stimulated with the super-agonist RQF peptide, they were capable of suppressing the proliferation of $CD4^+$ and $CD8^+$ T cells in response to HA Ag. This demonstrated that the 1E6 TCR could only confer Ag specific capabilities to a $CD4^+$ T_{REG} cells when the interaction between the introduced TCR and pMHCI was of a high affinity. This was in contrast to the study by *Plesa et al,* and suggests that although $CD4^+$ T_{REG} cells may possess a lower threshold for activation, the interaction between autoreactive MHCI restricted TCRs and pMHCI does not exceed this threshold. It is therefore possible to propose a new model for the requirements of an MHCI TCR to function in a $CD4^+$ T_{REG} cell that includes extremely low affinity autoreactive TCRs, which to date have not been used in this setting (Figure 4-30). From this study, it would be interesting to explore whether the up-regulation of CD69 can mark an MHCI TCR with the potential to re-direct the Ag specificity of a $CD4^+$ T_{REG} cell. This would allow for much quicker and easier testing of a panel of autoreactive MHCI restricted TCRs for their suitability in this approach. It would therefore be extremely worthwhile to determine whether $CD4^+$

T_{REG} cells transduced with the NY-ESO-1 TCR could up-regulate CD69 in response to the NY-ESO1 peptide.

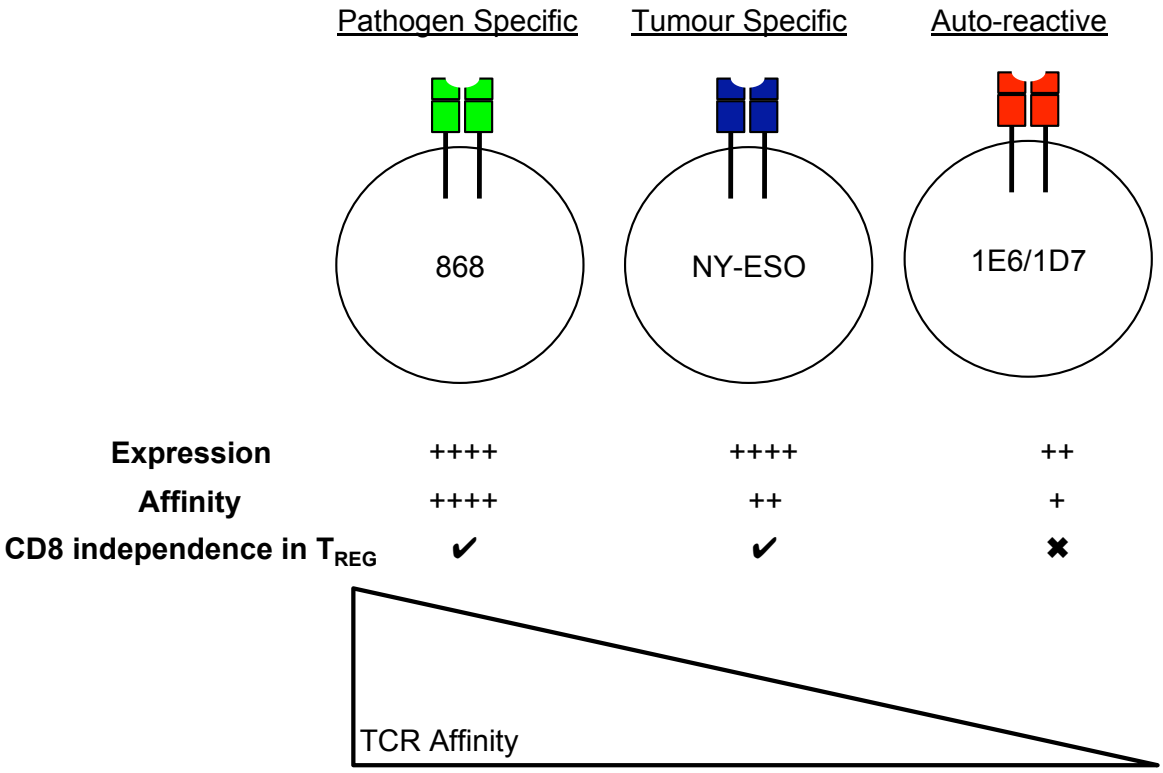

Figure 4-30 Model for MHCI restricted TCR Requirements in $CD4^+$ T_{REG} cells. MHCI TCRs can be split into three groups of pathogen specific, tumour specific and autoreactive TCRs. Both pathogen specific and tumour specific TCRs tend to be highly expressed (++++) when transduced into a $CD4^+$ T_{REG} cells and they tend to be of a high (++++) to medium-low (++) affinity for cognate Ag. This enables them to effectively re-direct the Ag specificity of a $CD4^+$ T_{REG}. Autoreactive TCRs tend to be weakly expressed (++) and have a low affinity for index peptide (+), which results in an overall low avidity interaction that may be dependent on the presence of CD8 co-receptor.

During a number of T_{REG} cell expansions, this study observed a reduction in the expression of FoxP3 as well an outgrowth of contaminating effector T cells. This led to a number of transduced T_{REG} cell expansions collapsing or being too low purity for use in Ag specific suppression assays. Additionally, there was inter-donor variation in the ability of RQF stimulated $1E6^+$ T_{REG} cells to suppress the proliferation of $CD4^+$ T cells. To improve both the phenotype and suppressive function of expanded $CD4^+$ T_{REG} cells, RAPA was added to subsequent cultures.

The maintenance of FoxP3 expression was determined in the RAPA expanded transduced cells after each round of expansion. Surprisingly, by the end of the third round of expansion the FoxP3 levels had dropped to below 70%. However, due to a limitation in the number of $CD4^+$ T_{REG} cells isolated in this study, it was not possible to perform a side-by-side comparison of non-RAPA expanded and RAPA expanded T_{REG} cells. Therefore, it could not be determined what the FoxP3 expression of these donors' T_{REG} cells would have been had they not been expanded in RAPA. Despite this observed reduction in FoxP3 expression, the $1E6^+$ T_{REG} cells were potent suppressors in an Ag specific suppression assay.

$1E6^+$ T_{REG} cells stimulated with the super agonist RQF peptide were capable of suppressing >80% of the proliferation of HA specific $CD4^+$ and $CD8^+$ T cells. This was much higher than in previous experiments using non-RAPA expanded T_{REG} cells. Due to this high percentage suppression an experiment to examine the potency of the RAPA expanded $1E6^+$ T_{REG} cells was performed. The ratio of PBMCs to T_{REG} cells that had previously been used was 5:1 and therefore in this experiment the ratio of PBMC to T_{REG} cells was titrated down to 40:1. It was clearly shown that at this low ratio of PBMC to T_{REG} cells the $1E6^+$ T_{REG} cells were still capable of suppressing, in an Ag specific manner, >50% of HA specific $CD4^+$ and >35% of HA specific $CD8^+$ T cell proliferation. In this experiment, the percentage suppression of "non-Ag specific" suppression was calculated and compared to the percentage suppression of Ag specific suppression. This revealed, that for the RAPA expanded $1E6^+$ T_{REG} cells to suppress 50% of the proliferation of HA specific $CD4^+$ and $CD8^+$ T cells, then estimated ratios of 8:1 and 6:1 PBMCs:T_{REG} cells respectively were required. However, for the same percentage suppression, $1E6^+$ T_{REG} cells that were stimulated with RQF peptide were required at lower T_{REG} cells ratios of 40:1 and 20:1. These data clearly demonstrated the benefit of Ag specific over polyclonal T_{REG} cells.

In conclusion to this section of this study, we have demonstrated that the two autoreactive MHCI TCRs were weakly expressed when transduced into primary

human T cell populations. This weak expression and overall low affinity for cognate peptide culminated in an overall inability to signal when introduced into primary human T cell populations. Therefore, it can be concluded that for low affinity MHCI TCRs to re-direct the Ag specificity of a $CD4^+$ T_{REG} cells they will require further optimisation such as an improvement of expression, affinity for cognate peptide or co-expression of the CD8$\alpha\beta$ co-receptor.

5. Approaches to Enhance 1E6 TCR Function in Human T_{REG} Cells

5.1. Introduction

TCR signalling is initiated by TCR recognition and binding to pMHC on the surface of APCs. For an MHCI restricted TCR, the binding to pMHCI is stabilised by the CD8 co-receptor. The stability provided by the co-receptor is integral to low affinity TCR-pMHC interactions, but dispensable for high affinity interactions (Holler and Kranz, 2003). Additionally, the productive hit rate model of T cell activation infers that the threshold for T cell activation is the product of multiple TCR-pMHC interactions (Valitutti et al., 1995). Therefore, the ability of an MHCI TCR to transfer Ag specificity to a $CD4^+$ T cell is influenced by the high avidity interaction between TCR and pMHC and it appears that there is a threshold below which productive signalling will not occur, even in T_{REG} cells. Theoretically, there are four methods that can be used to improve the functional avidity between transferred TCRs and pMHC (Figure 5-1):

1. Engineering of the hypervariable CDR3 regions of the TCR to increase TCR-pMHC binding affinity
2. Engineering altered peptide ligands that bind TCR with a greater affinity.
3. Increasing the expression level of the TCR to promote enhanced TCR serial triggering.
4. Increasing the stabilisation of TCR-pMHCI interactions by introducing the CD8 co-receptor into $CD4^+$ T cells.

The alteration of CDR3 regions requires PCR directed mutagenesis of this region in both the TCRα and TCRβ chains and the ability to screen mutated TCRs for enhanced pMHCI binding. As the methods involved in CDR3 engineering are highly time-consuming, this method was beyond the scope of this study. Furthermore, others have shown the dangers of re-directing T cell Ag specificity using altered TCRs, as increasing a TCRs' affinity for a single peptide can increase

the risks of T cell off target toxicity (Cameron et al., 2013; Linette et al., 2013). The second method of using altered peptide ligands has already been explored in Chapters 3 and 4 with the use of the high affinity RQF peptide, which activates the 1E6 TCR in a CD8 independent manner. Furthermore, whilst this approach is useful for proof of concept studies, it lacks translational potential. Therefore, this Chapter will concentrate of the two final mechanisms of enhancing autoreactive MHCI restricted TCR function in T_{REG} cells.

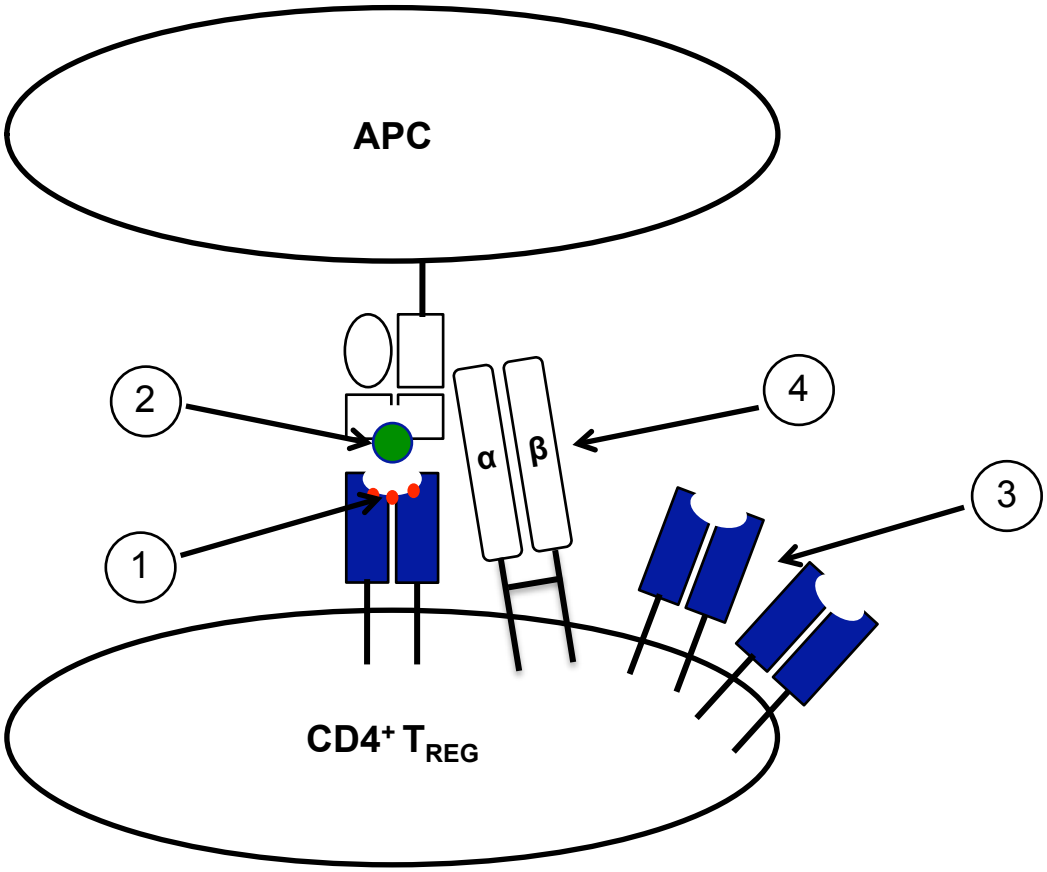

Figure 5-1 Mechanisms to enhance autoreactive MHC Class I TCR function in human T_{REG} cells. Four potential mechanisms can be used to improve the function of MHCI TCRs in human $CD4^+$ T_{REG} cells. 1) engineering of the TCR CDR3 region, 2) The use of altered peptide ligands with enhanced affinity for TCR 3) increased expression of introduced TCRs on the surface of T_{REG} cells and 4) the introduction of the CD8 co-receptor on the surface of T_{REG} cells.

In theory, the 1E6 TCR should be able to function in a CD8 independent manner as evidenced by the data already shown using the super agonist RQF peptide to activate this TCR. It was observed that the 1E6 TCR was expressed at a low level on the surface of all primary human T cell populations, which may explain why the transfer of the 1E6 TCR could not re-direct the Ag specificity of $CD8^+$ T cells towards the ALW peptide. The reduced TCR expression, combined with a low affinity for pMHC, may lead to the overall TCR-pMHC interaction being of too low an avidity to activate the T cell. Therefore, increasing the expression of the 1E6 TCR on the surface of primary human T cells may rescue the ability of this TCR to respond to the ALW peptide, by increasing the available number of TCRs that can be triggered by pMHCI. The main hypothesis for poor expression of introduced TCRs is a low interchain affinity of the TCRα and TCRβ – this can lead to reduced pairing of TCR chains and a reduced ability to compete for CD3 molecules. In the past, multiple methods have been identified to improve interchain affinity and subsequently the expression of exogenous TCRs. Two of these have already been incorporated into the vector expression system of the 1E6 TCR – 1) codon optimisation of TCR genes to improve translation and production of TCR protein (Scholten et al., 2006) and 2) the use of 2A linker sites between TCRα and TCRβ chains to improve pairing of introduced chains (Leisegang et al., 2008). Other potential modifications that have shown to improve expression of the exogenous TCRs, include the incorporation of a second disulphide bond between the constant domains of the TCRα and TCRβ chains to improve pairing (Cohen et al., 2007) and the removal of TCR glycosylation sites, which has been shown to improve the functional avidity of re-directed T cells (Kuball et al., 2009). However, these have not been tested for the 1E6 TCR in this thesis. The inclusion of the second disulphide bond was incorporated into the 1D7 TCR, however as observed in Chapters 3 and 4, the expression of this TCR was still compromised.

A somewhat surprising route identified to improve the expression of weak TCRs was the replacement of human TCRα and TCRβ chain C regions with the murine equivalent (Cohen et al., 2006). These human-murine (“murinised”) hybrids led to

an over-expression of the TCRs in comparison to fully human TCRs, due to a preferential pairing of murine C domains and an overall improved TCR-CD3 stability (Cohen et al., 2006). However, the adoptive transfer of T cells expressing murine TCRs led to some patients developing a humoral immune response against the xenogeneic gene sequences (Davis et al., 2010). In order to reduce the potential immunogenicity to "murinised" TCRs, the amino acids in the murine TCRα and TCRβ chains that are important for increased TCR expression have been identified (Sommermeyer and Uckert, 2010). Indeed, only 9 human to murine amino acid substitutions were found to be crucial in facilitating increased TCR expression, thus these minimal changes may reduce immunogenicity of these TCRs. In a similar vein, substitutions in the transmembrane domain of the human TCRα can improve the expression of introduced TCR chains. A full TCR-CD3 complex is first assembled in the endoplastic reticulum and then transported to the cell surface. The TCRα chain has been identified as the most instable chain of this complex and can decrease the efficiency of TCR$\alpha\beta$ pairing and it's coupling to CD3 molecules (Bonifacino et al., 1990; Kearse et al., 1994; Shin et al., 1993). Therefore, investigations were made as to whether TCRα chain stability could be improved by increasing the hydrophobicity of the TCRα transmembrane domain (Haga-Friedman et al., 2012). In this study it was found that three substitutions of mutable amino acids for hydrophobic leucine or valine amino acids could almost double the percentage of transduced cells that stained with specific pMHC multimer (Haga-Friedman et al., 2012).

The final method of improving the function of MHCI restricted TCRs in $CD4^+$ T cells, is the introduction of the CD8 co-receptor in these cells. The data from model cell lines in Chapter 3 suggested that Jurkat T cells transduced with the 1E6 TCR could signal in response to ALW peptide in the presence of high level CD8 expression. Therefore, the ability of the CD8 co-receptor to rescue the 1E6 TCR in human $CD4^+$ T_{REG} cells will be assessed. It was also determined in Chapter 4 that the 1E6 TCR was unable to re-direct the Ag specificity of $CD8^+$ T cells, although it is unclear whether this was due to poor 1E6 TCR expression, which culminated in

a low functional avidity. Consequently, the ability of a high affinity CD8 co-receptor to stabilise 1E6 TCR-pMHC interactions in T_{REG} cells will also be explored. The high affinity CD8 co-receptor has a single amino acid substitution in the CD8α chain that has been shown to enhance CD8 binding to HLA-A2 by up to 4-fold (Cole et al., 2007b). It is hypothesised that the use of a high affinity CD8 co-receptor may overcome reduced 1E6 TCR expression by increasing the dwell time between TCR and pMHC. To explore this mechanism, LV vectors will be used to co-express either the WT CD8$\alpha\beta$ or the high affinity CD8$\alpha\beta$-S53N co-receptor with the 1E6 TCR on the surface of $CD4^+$ T_{REG} cells.

The final section of this thesis will therefore incorporate the described techniques to enhance the signalling ability of the 1E6 TCR in response to the ALW peptide in $CD4^+$ T_{REG} cells.

5.2. Materials and Methods

Plasmids

The 1E6 variant TCR vectors, 1E6 LVL and 1E6 MMu were identical to the original pELNS.1E6.RatCD2 vector, apart from the amino acid substitutions described. Both these vectors were generated by and kindly provided to us by John Bridgeman (Cardiff University, UK). The CD8 expression vectors, pELNS.CD8β.IRES.CD8α and pELNS.CD8β.CD8αS53N were also generated by John Bridgeman (Cardiff University, UK) and were a kind gift from Linda Wooldridge (University of Bristol, UK).

Dual-Transduction of $CD4^+$ T_{REG} Cells

$CD4^+$ T_{REG} cells were transduced with two LV vectors concurrently to obtain cells co-expressing genes encoded from each vector. As with single transduction, each LV was titrated, as previously described, to achieve a value of transducing units/ml (TU/ml). 2 $x10^5$ T_{REG} cells were activated with αCD3/CD28 beads and IL-2 for 48 hours as described previously (Chapter 4.2). Each LV was added to $2x10^5$ cells in

the same Eppendorf at a TU/cell of 5TU/cell and cells were spin infected as previous. Therefore, the total LV titre added to the T_{REG} cells was 10TU/ml.

Ag Specific Suppression Assay

Autologous cryopreserved PBMCs were thawed and labelled with Cell Trace Violet. The cells were re-suspended to $1x10^6$/ml and 100μl cells were aliquoted per well of a 96 well u-bottom plate. HA Ag was used to stimulate the PBMCs at a final concentration equivalent to 180ng/ml HA. Transduced and expanded 1E6$^-$CD8-S53N$^+$, 1E6$^+$CD8-S53N$^+$ and 1E6$^+$CD8-S53N$^-$ T_{REG} cells were thawed from liquid N_2, washed and re-suspended to $2x10^5$/ml. 10,000 T_{REG} cells were added to each well in 50μl for a final PBMC:T_{REG} cell ratio of 10:1. ALW and RQF peptide were added to each well at a final concentration of 1μg/ml. The assay was set up in triplicate wells, with one triplicate per condition. The cells were incubated for 6 days at 37°C 5% CO_2. 100μl of supernatant from each well was retained for IFNγ ELISA and stored at -80°C. The triplicate wells were harvested into a single FACS tube and stained with fluorescently labelled antibodies for expression of CD3, CD4, Rat CD2 and CD8. 7AAD was included to exclude dead cells from analysis and proliferation of responding CD4$^+$ and CD8$^+$ T cells was measured by loss of Cell Trace Violet.

IFNγ Enzyme-Linked Immunosorbant Assay (ELISA)

IFNγ ELISAs were performed using an ELISA MAX IFNγ set (Biolegend, UK). Briefly, 96 well Maxisorp plates (Nunc, UK) were coated with 50μl pre-titrated IFNγ capture antibody and incubated at 4°C overnight. The following day the antibody was removed and excess protein binding sites blocked for 1 hour at room temperature in 200μl blocking buffer (PBS 1% bovine serum albumin (BSA, Sigma Aldrich, UK)). The plates were then washed 4 times in wash buffer, PBS 1% Tween (Tween, Cytotech, Denmark) using a 12 channel Nunc Immunoplate washer (Nunc, UK). 50μl of culture supernatant from the Ag specific suppression assay was diluted 1:10 in assay buffer (PBS 1% BSA) and added to the plate. To quantify the amount of IFNγ release, recombinant protein standards were added to

duplicate wells at a range of doubling dilutions from 2000pg/ml to 31.3pg/ml. The supernatants and standard were incubated for a further 2 hours at room temperature, the plates were washed four times and 50μl of biotinylated detection antibody, diluted 1:200 in assay buffer was added to the wells for 1 hour at room temperature and then removed. 50μl of avidin HRP, diluted 1:1000 in assay buffer, was added to the wells for 30 minutes at room temperature in the dark and the plates washed a further five times. 50μl of substrate solution, equal volumes of stabilised Hydrogen Peroxide and stabilised tetramethylbenzidine (R&D, UK) were added to the wells and the assay left to develop for 20-30 minutes at room temperature in the dark. The reaction was stopped using 50μl of 1M hydrosulphuric acid and the absorbance of each well measured at 450nM using a microplate spectrophotometer (Biorad, Hertfordshire) A standard curve was generated using Microsoft excel from the optical density (OD) values of the dilution of recombinant IFN-γ protein. The unknown values were calculated using the equation of the straight line y=mx+c where y is the OD value, m the gradient of the line, c the y intercept and x the unknown quantity.

5.3. Results

5.3.1. Minimal Amino Acid Modifications of the 1E6 TCR can Increase its Expression in Primary Human T Cells.

To investigate whether improving the expression of the 1E6 TCR could rescue its response to the ALW peptide, two variants, that have minor amino acid substitutions in either the C regions or transmembrane region of the TCR were obtained. The 1E6 LVL variant caries three amino acid substitutions in the transmembrane domain of the TCRα chain that increases its' hydrophobicity (Figure 5-2 (A)). The increase in hydophobicity has been shown to improve the expression of a number of exogenously expressed MHCI restricted TCRs by increasing the stability of the TCRα chains (Haga-Friedman et al., 2012). The 1E6 MMu variant has four and five human to murine amino acid substitutions in the C regions of the 1E6 TCRα and TCRβ chains respectively (Figure 5-2 (B)). Although

it has been shown that these substitutions do not increase the exogenous expression of TCRs to the same extent as "murinised" TCRs, the minimal substitutions are hypothesised to decrease any immunogenicity of these TCRs (Sommermeyer and Uckert, 2010).

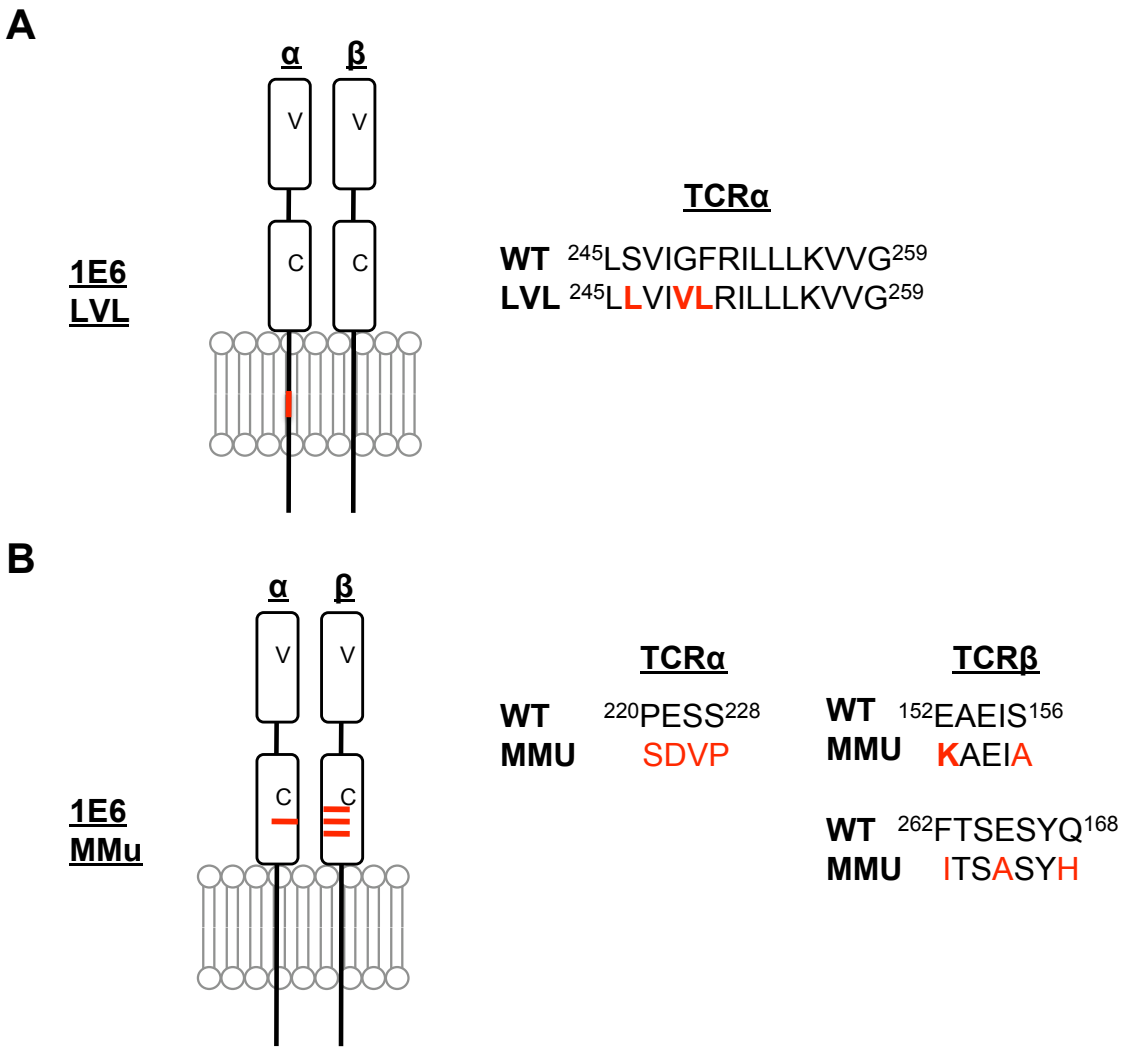

Figure 5-2 Schematic Representation of Substitutions in the Variant 1E6 TCRs. Sequence alignments are shown indicating the position of variants in the (A) transmembrane domain of the 1E6 TCRα chain for the 1E6 LVL variant and (B) C region of the 1E6 MMu variant. The amino acid substitutions are highlighted in red.

To first test the expression of 1E6 LVL and 1E6 MMu variants in comparison to the 1E6 (from herein designated 1E6 WT) TCR, TCRαβ$^+$ Jurkat cells were transduced with LV encoding one of the three TCRs. TCRαβ$^+$ Jurkat cells were used to ensure

there would be competition for cell surface expression between the introduced and endogenous TCRs. However, in contrast to the transduction of primary T cell populations, all transduced TCR$\alpha\beta^+$ Jurkat cells expressed TCRvβ8, with only a proportion of these cells expression Rat CD2 (Figure 5-3 (A)). This was a very similar pattern of expression observed when TCR$\alpha\beta^-$ Jurkat cells were transduced, as seen in Chapter 3. Therefore, differences in expression between 1E6 variant TCRs could only be measured by assessing the MFI of TCRvβ8 expression. However, no significant enhancement of TCRvβ8 expression was observed in comparison of the 1E6 variant and WT TCRs.

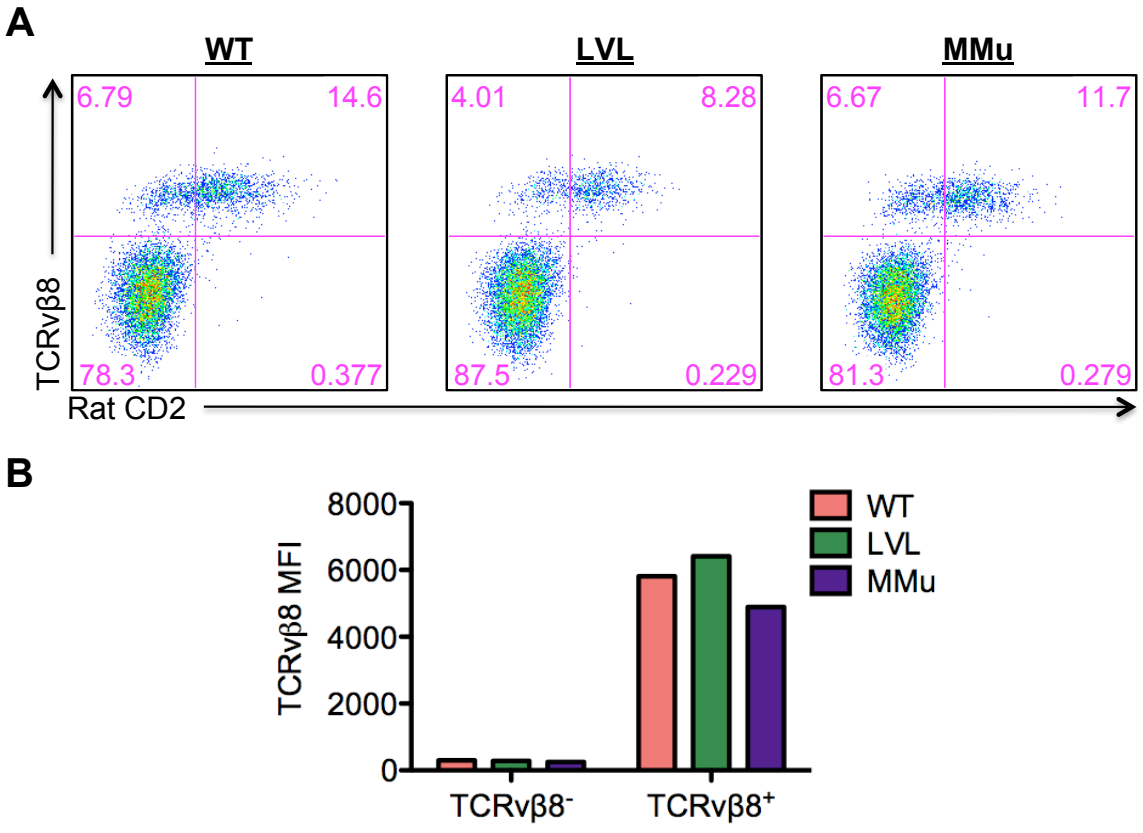

Figure 5-3 Expression of 1E6 Variant TCRs in a TCR$\alpha\beta^+$ Jurkat Cell Line. LV encoding the 1E6 WT, LVL and MMu variant TCRs were transduced into TCR$\alpha\beta^+$ NFB2-1 Jurkat cell lines. (A) Transduced Jurkat cell lines were stained with TCRvβ8 and Rat CD2 fluorescently labelled antibodies and analysed by FACS. (B) The MFI of TCRvβ8 expression was assessed in TCRvβ8$^-$ and TCRvβ8$^+$ cell populations.

It is possible that the lack of difference between the expression of the 1E6 variants in Jurkat cells was due to the endogenous Jurkat cell TCR providing little to no competition for the introduced 1E6 TCRs. Therefore, to determine whether transduction with the 1E6 TCR variants could lead to an increased expression of the 1E6 TCR on transduced primary cells, $CD4^+$ T_{EFF} cells were transduced with either the 1E6 WT, 1E6 LVL or 1E6 MMu TCR. $CD4^+$ T_{EFF} cells were chosen over $CD4^+$ T_{REG} cells due to their abundance and the assumption that competition between TCRs would not significantly differ between T_{EFF} and T_{REG} cells. $CD4^+$ T_{EFF} cells were therefore isolated from three healthy control donors and transduced with 1E6 WT, 1E6 LVL or 1E6 MMu encoding LV. The transduced cells were then stained for TCRvβ8 and Rat CD2 expression 72 hours post transduction. These analyses showed that in two of three cases for LVL and in all cases for MMu, the substitutions in the 1E6 TCR resulted in a greater proportion of transduced cells that co-express the 1E6 TCRvβ8 and Rat CD2 (Figure 5-4 (A)). Additionally, the 1E6 MMu variants led to a significant increase in the co-expression of TCRvβ8 and Rat CD2 in comparison to the 1E6 WT TCR (Figure 5-4 (C)). Furthermore, in the three donors, transduction with the 1E6 MMu construct also led to a higher level of TCRvβ8 expression on the Rat $CD2^+$ transduced cells, compared to the 1E6 WT construct (Figure 5-4 (E)).

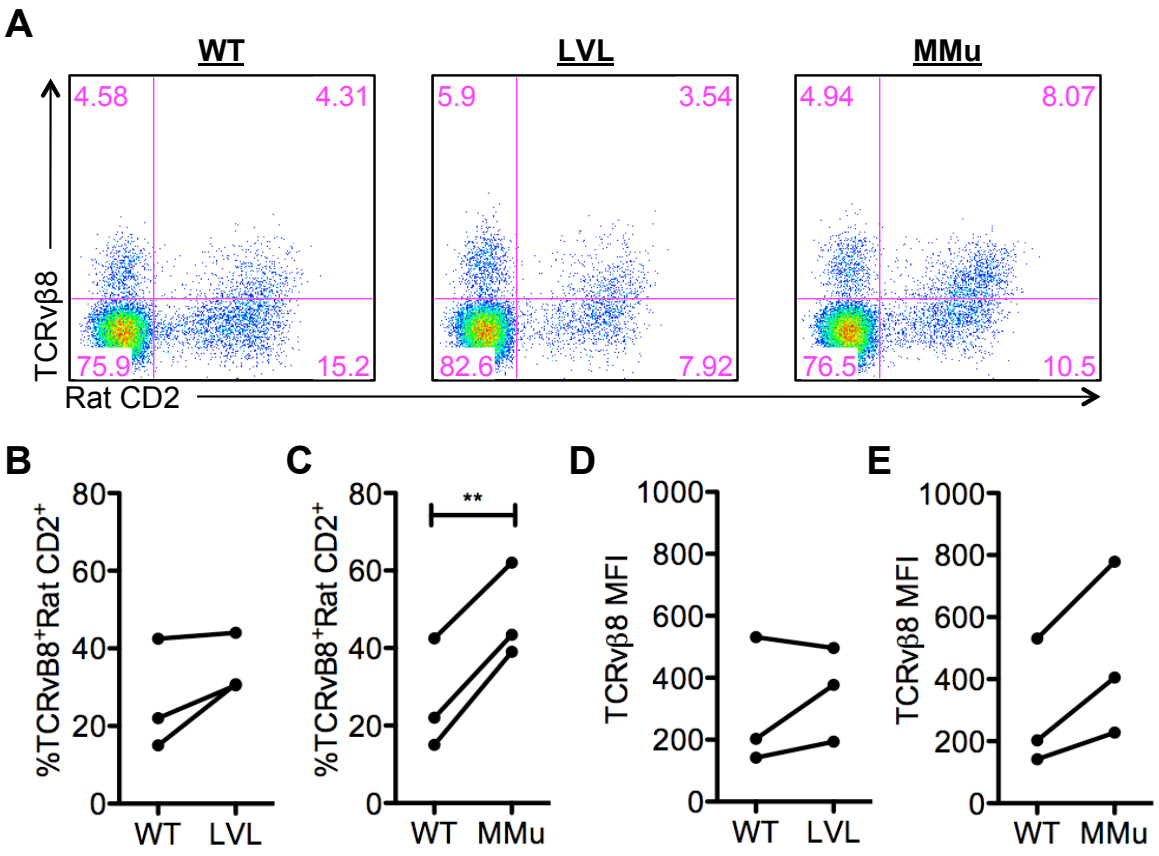

Figure 5-4 Expression of 1E6 Variant TCRs in $CD4^+$ T_{EFF} Cells. $CD4^+$ T_{EFF} cells were transduced with LV encoding the different 1E6 variant TCRs. (A) FACS plots show 1E6 variant TCR, transduced cells stained for expression of TCRvβ8 and Rat CD2. (B) The percentage of dual expressing TCRvβ8 and Rat CD2 transduced cells was compared between $CD4^+$ T_{EFF} cells transduced with 1E6 WT and 1E6 LVL TCR and (C) 1E6 WT and 1E6 MMu TCR. (D) The MFI of TCRvβ8 of total $RatCD2^+$ cells was compared between $CD4^+$ T_{EFF} cells transduced with 1E6 WT and 1E6 LVL TCR and (E) 1E6 WT and 1E6 MMu TCR. Statistical significance was calculated using a paired Student's t test ** = $p < 0.01$. Each pair represents transduction in a single donor.

5.3.2. Increased Expression of the 1E6 TCR on the Surface of T_{REG} Cells is Insufficient to Rescue the T_{REG} Cells Response to WT Peptide

Given the promising results with the 1E6 variant TCRs in T_{EFF} cells, we sought to transduce T_{REG} cells with each 1E6 TCR variant to test the signalling capability of each TCR in response to ALW peptide. This was tested in $CD4^+$ T_{REG} cells as the signalling requirements are thought to be different between these two cell subsets, as discussed previously (Plesa et al., 2012). $CD4^+$ T_{REG} cells from a single donor were isolated, activated, split into three groups and transduced with either the 1E6 WT TCR or one of the two 1E6 variant TCRs (Figure 5-5 (A)). Similar to the profile seen post transduction of $CD4^+$ T_{EFF} cells, transduction with the 1E6 TCR variants yielded a higher percentage of Rat $CD2^+$ cells co-expressing TCRvβ8 with percentages of 61% for 1E6 LVL and 70% for 1E6 MMu compared to 47% for 1E6 WT TCR (Figure 5-5 (B)). The overall TCRvβ8 MFI of T_{REG} cells transduced with 1E6 MMu was also higher compared to T_{REG} cells transduced with 1E6 LVL and 1E6 WT (Figure 5-5 (C)). To determine whether this increased expression of the 1E6 TCR led to a rescue in its response to ALW peptide, the ability of transduced cells to up-regulate CD69 in response to peptide was measured. Transduced cells were incubated with autologous PBMCs that had been pulsed with PBS, ALW or super-agonist peptide RQF for 16 hours, as with previous experiments. The expression of CD69 was then measured in Rat $CD2^+$ populations. This analysis revealed that there was no difference in the response of $CD4^+$ T_{REG} cells to the ALW peptide when cells were transduced with the 1E6 higher expression variants (Figure 5-5 (C)). There was however a significant increase in the percentage of cells that up-regulated CD69 in response to the RQF peptide when T_{REG} cells transduced with the LVL or MMu TCRs were stimulated (Figure 5-5 (D)).

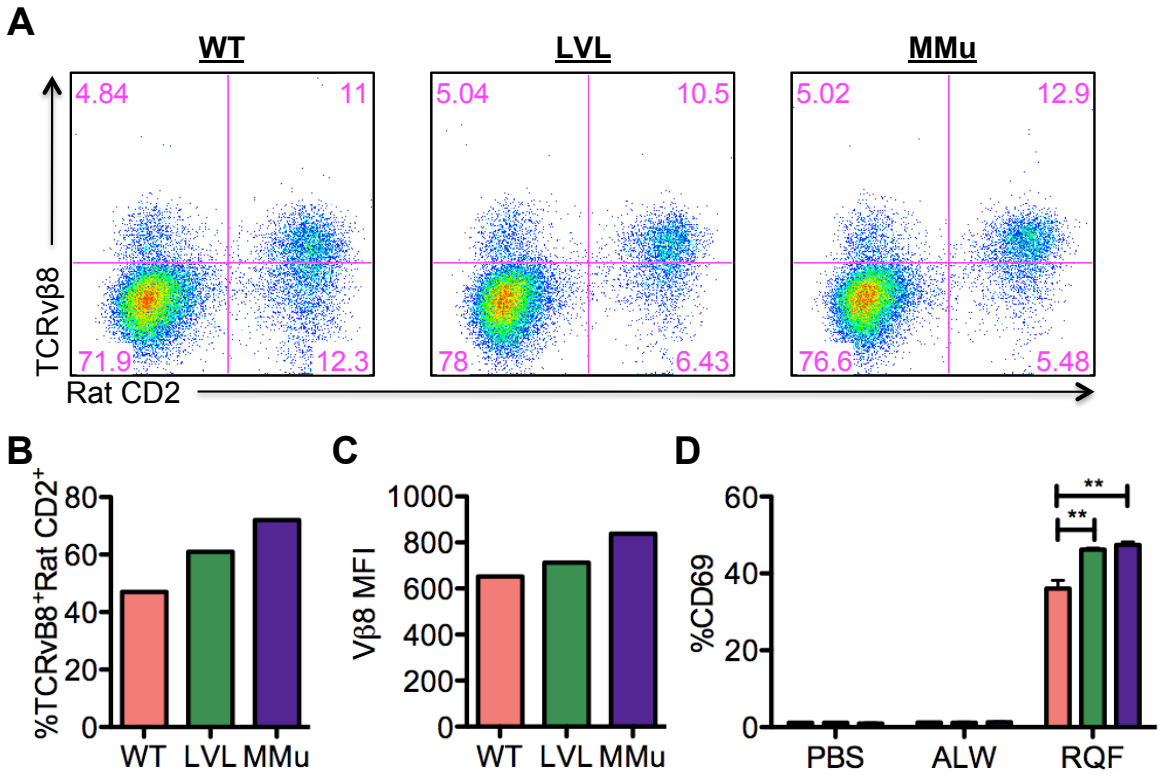

Figure 5-5 Increased expression of TCRvβ8 in $CD4^+$ T_{REG} cells fails to rescue the 1E6 TCR response to the ALW peptide. $CD4^+$ T_{REG} cells from a single donor were transduced with LV encoding one of the three 1E6 Variant TCRs. (A) Expression of TCRvβ8 and Rat CD2 was assessed by flow cytometry in $CD4^+$ T_{REG} cell transduced with either the 1E6 WT, LVL or MMu variants. (B) The Percentage of TCRvβ8$^+$Rat $CD2^+$ cells for T_{REG} cells transduced with each 1E6 variant TCR and (C) the MFI of TCRvβ8 expression in total Rat $CD2^+$ cells for each variant was assessed. (D) The transduced populations shown were stimulated with autologous PBMCs that had been pulsed with either PBS, WT peptide ALW or RQF peptide. Expression of CD69 was assessed in all Rat $CD2^+$ cells under each stimulation condition. Data shown is from one healthy control donor. Statistical significance was calculated using an unpaired Student's t test. ** = P<0.01.

5.3.3. Transduction of Jurkat Cells with CD8αβ Co-receptor and a High Affinity Variant, can Rescue the 1E6 TCRs Response to WT Peptide.

In the previous experiments, it was determined that the increased expression of the 1E6 TCR on $CD4^+$ T_{REG} cells was not sufficient to re-direct the Ag specificity of these cells to the ALW peptide. It was hypothesised, that to fully re-direct the Ag specificity of $CD4^+$ T_{REG} cells towards the ALW epitope, the expression of the CD8αβ co-receptor would be required. As previously mentioned, for productive TCR signalling sufficient numbers of TCRs must be triggered and each triggering must be within the optimal dwell time for TCR-pMHC interactions (Kalergis et al., 2001; Valitutti et al., 1995). Therefore the expression of CD8αβ may be able to increase the dwell time between TCR-pMHC interactions and subsequently lead to productive signalling. From experiments in model cell lines in Chapter 3, it was observed that CD8α expression on Jurkat cells could rescue the 1E6 TCRs' response to ALW peptide. However, the ability of CD8α to rescue the response only occurred when CD8α was highly expressed on a transfected cell line (J76CD8α) and not when it was co-expressed with the 1E6 TCR via the same LV construct. This suggested that to achieve full expression of CD8α, this gene would need to be expressed on a separate construct to the 1E6 TCR. Therefore, a separate LV vector that encoded the full CD8αβ co-receptor was obtained, a kind gift from Professor Linda Wooldridge (Figure 5-6 (A)). Furthermore, results from Chapter 4 also demonstrated that transduction with the 1E6 TCR was unable to re-direct the Ag specificity of primary $CD8^+$ T cells towards the ALW peptide. Thus, a second construct encoding a high affinity CD8αβ co-receptor variant was obtained. This high affinity CD8αβ variant contains a serine to arginine substitution at amino acid 53 of the CD8α chain and has been designated CD8βαS53N (Figure 5-6 (B)) (Cole et al., 2007b). The ability of both the CD8βα WT co-receptor and CD8βαS53N co-receptor to rescue the 1E6 TCR could therefore be tested simultaneously. For simplicity in all following experiments, cells transduced with the CD8αβ WT construct will be referred to as CD8-WT^+ and those transduced with the CD8βαS53N co-receptor as CD8-$S53N^+$.

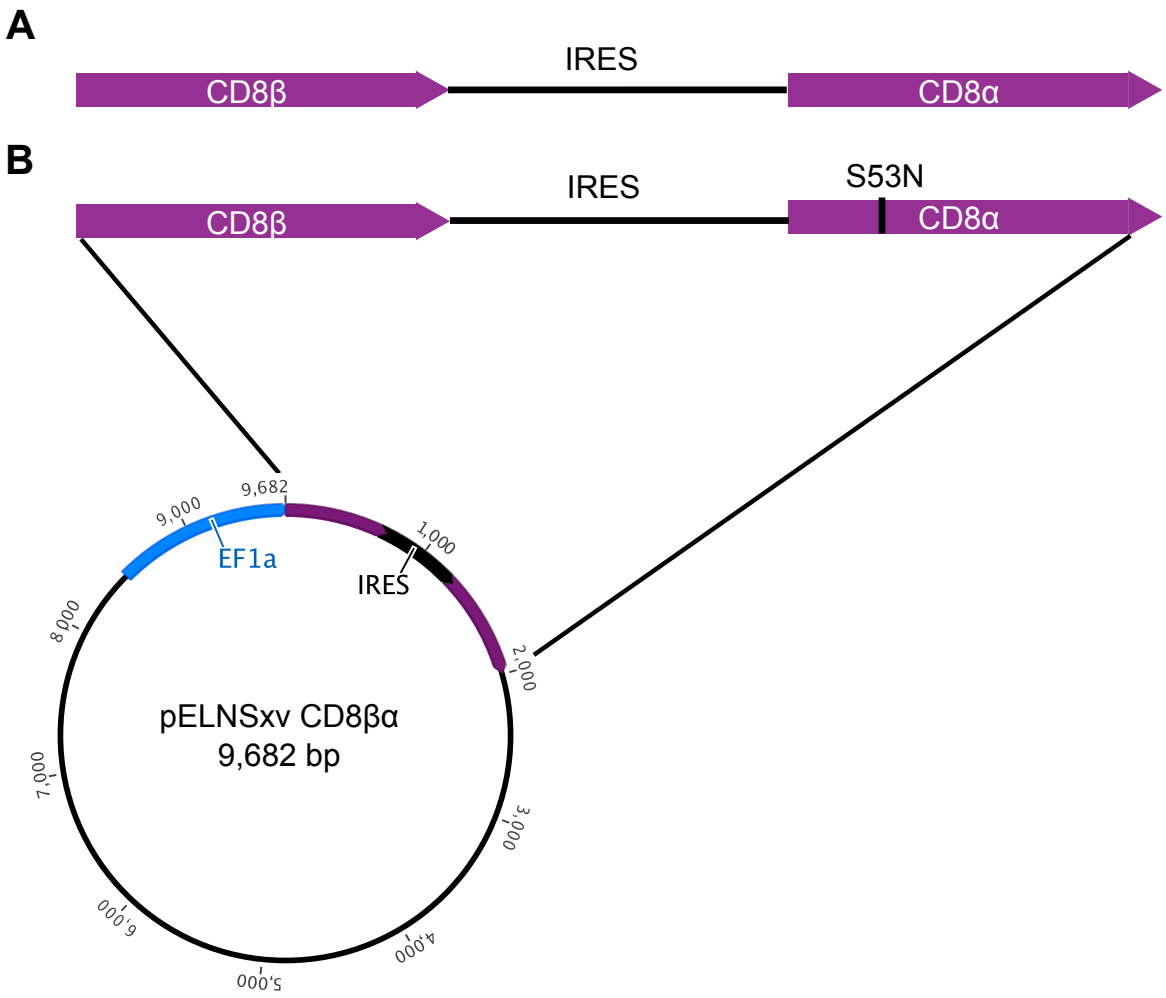

Figure 5-6 CD8αβ Expression Plasmids. The CD8α and CD8β chains were cloned into the pELNS LV vector under the control of the EF1α promoter with an IRES site separating the two chains (A) A schematic of the CD8βα WT genes inserted into the vector (B) A schematic of the CD8βαS53N variant depicting the position of the S53N substitution in the CD8α chain.

The ability of both the CD8 co-receptors to rescue the 1E6 TCR was first examined using Jurkat cell lines. The TCR$\alpha\beta^-$ Jurkat cell line J76, which had previously been transduced with the 1E6 TCR, were transduced for a second time with either the CD8-WT LV or the CD8-S53N LV. J76CD8α cells that had been transduced with the 1E6 TCR served as a positive control for the following experiments. J76CD8α cells, that had previously been transfected to express CD8α were transduced with the 1E6 TCR for the following experiments and therefore were all CD8α^+ but only 20% 1E6 TCR$^+$ at the time of staining, due to a low transduction efficiency. The transduced cell lines were stained using CD8α and TCRvβ8 fluorescent antibodies and a clear population of cells that co-expressed both receptors was observed (Figure 5-7 (A)). Similar to results seen in Chapter 3, expression of CD8-WT and CD8-S53N was lower in the LV transduced cells compared to CD8α expression on the transfected Jurkat cell line. The transduced cells were stained with an ALW-HLA-A2 multimer to determine if the introduced CD8 co-receptors could stabilise the 1E6 TCR-pMHC interaction. The results of the multimer staining revealed that 20% of cells expressing both the 1E6 TCR and the CD8-S53N could be stained with the ALW-MHCI multimer (Figure 5-7 (B)). In contrast, only 3% of cells co-expressing the 1E6 TCR and the CD8-WT co-receptor stained with the same MHCI multimer. As observed in Chapter 3, 40% of 1E6$^+$J76CD8α cells stained with the ALW-MHCI multimer and little staining was observed in CD3$^-$ cells (Figure 5-7 (B)). The staining of the 1E6 TCR$^+$ cells lines with the ALW-MHCI multimer was specific, as no staining was observed with an irrelevant peptide control VMN multimer (Figure 5-7 (C)).

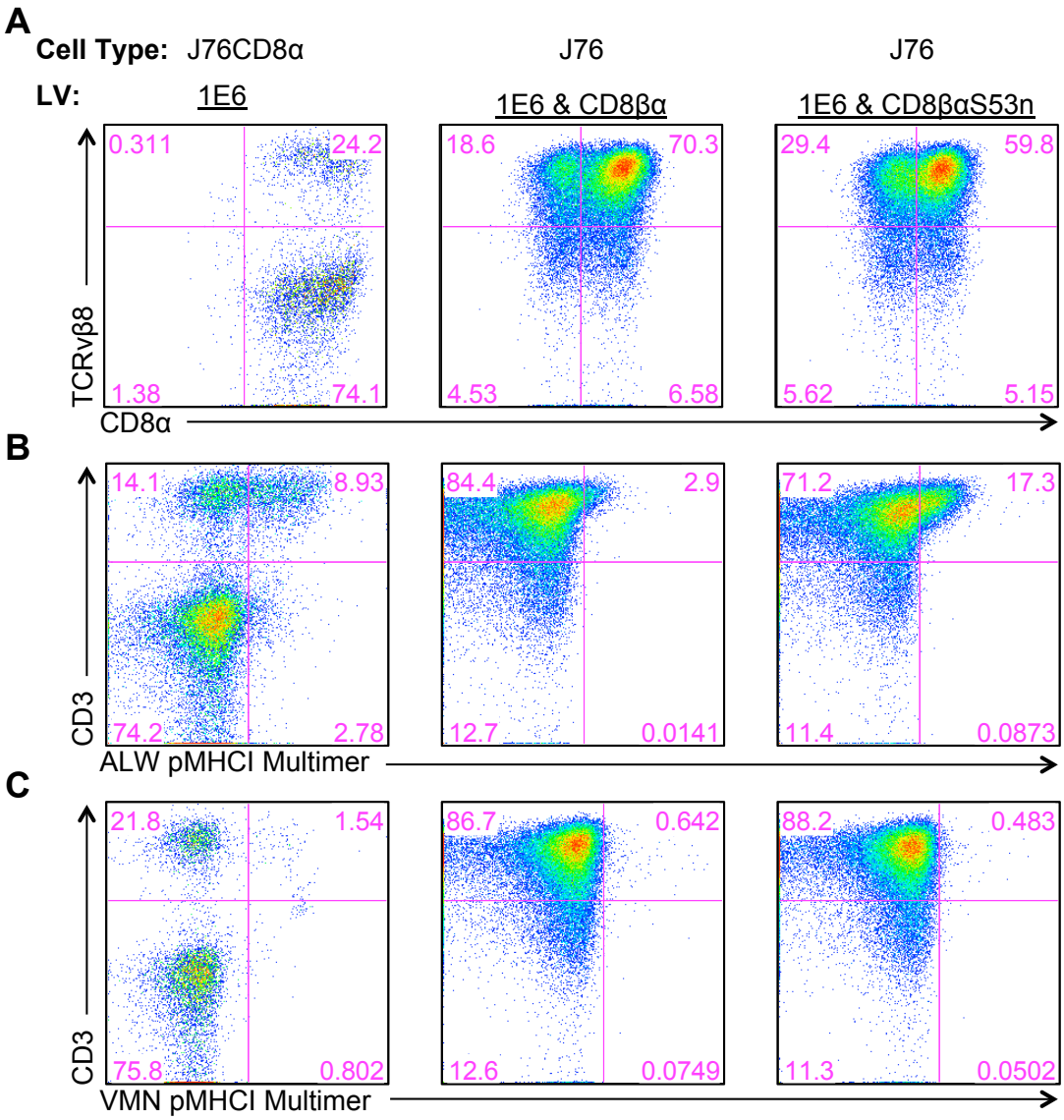

Figure 5-7 Expression of CD8αβ Genes via LV Transduction in 1E6$^+$ J76 cells. 1E6$^+$ J76 cells were transduced with LV encoding either the CD8-WT or the CD8-S53N co-receptor. (A) To assess for successful transduction of 1E6$^+$ J76 cells with the two CD8 expression vectors, the cells were stained with antibodies against TCRvβ8 and CD8α. 1E6 transduced J76CD8α cells were stained as a positive control (B) The CD8 expressing, 1E6$^+$ Jurkat cell lines were stained with an αCD3 antibody and an MHCI multimer in complex with ALW peptide (C) or a VMN pMHCI multimer, as an irrelevant multimer control. The 1E6$^+$J76CD8α cell lines were stained as a positive control.

Having established that co-expression of CD8-S53N increased ALW-MHCI multimer binding in at least a proportion of 1E6 expressing Jurkat cells, it was investigated if expression of this co-receptor could increase the 1E6 TCRs response to ALW peptide in a peptide activation assay. In this experiment, 1E6

transduced J76 ($1E6^{+}$J76) cells served as a negative control and 1E6 transduced J76CD8α ($1E6^{+}$J76CD8α) as a positive control. All transduced cell lines were FACS sorted so 100% of cells in culture had the desired phenotype. All three CD8 expressing cell lines were capable of responding to PBMCs pulsed with the ALW peptide (Figure 5-8 (A)). Although a clear hierarchy in the level of response was observed, with stimulation of $1E6^{+}$J76CD8α yielding the greatest response, followed by $1E6^{+}$CD8-$S53N^{+}$ J76 cells and then $1E6^{+}$CD8-WT^{+} J76 cells. As before, the $1E6^{+}$J76 cells were unable to respond to PBMCs pulsed with ALW peptide. All four population of cells responded to PBMCs pulsed with the RQF peptide, with $1E6^{+}$J76CD8α yielding the highest response (Figure 5-8 (B)).

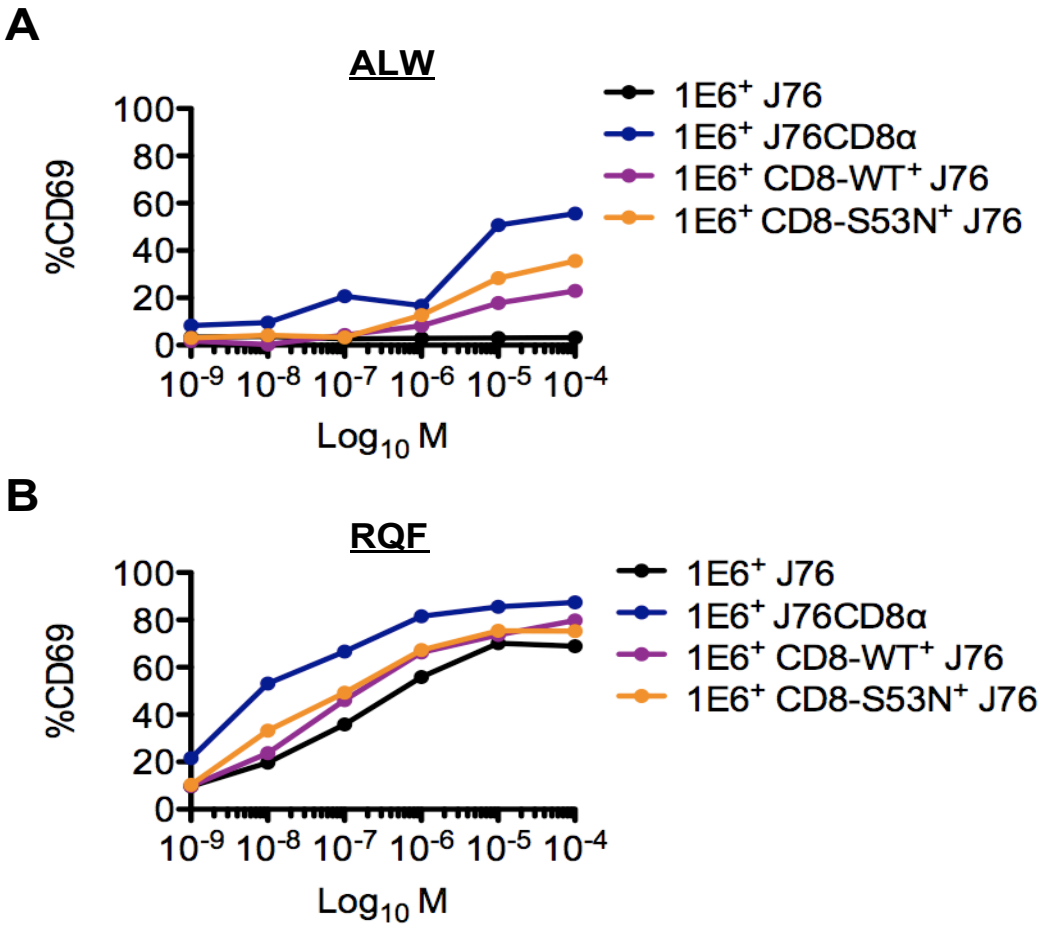

Figure 5-8 Co-expression of the 1E6 TCR and CD8αβ co-receptors re-direct the specificity of Jurkat cells. The cell lines indicated were sorted to a high purity of transduced cells and incubated with PBMCs pulsed with log dilutions of (A) ALW or (B) RQF peptide and the up-regulation of CD69 was assessed after 16 houra. Data is representative of one experiment.

5.3.4. $CD4^+$ T_{REG} cells can be Co-Transduced to Express Both CD8-S53N and the 1E6 TCR

The results observed in model cell lines suggest that transduction of $CD4^+$ T_{REG} cells with CD8-S53N would have the best chance of aiding the 1E6 TCR in re-directing the Ag specificity of these cells. For expression of both the 1E6 WT TCR and CD8-S53N co-receptor, $CD4^+$ T_{REG} cells were co-transduced with both LV vectors. Others have shown success with co-transduction of high titre, VSV-G pseudotyped LV's when cells are infected with each LV at the same time (Frimpong and Spector, 2000). Therefore, T_{REG} cells were isolated from a healthy control donor, T02 and after 48 hours of aCD3/CD28 stimulation, both the 1E6 LV and the CD8-S53N LV were added to the T_{REG} cells and spin infected as previously described. The co-transduction of the cells was then characterised 72 hours post transduction. The co-transduced cells were stained with α-TCRvβ8 and α-Rat CD2 antibodies to confirm transduction with the 1E6 WT TCR (Figure 5-7 (A)). The cells were also stained with α-CD8α and single populations of CD8α expressing cells and TCRvβ8 expressing cells were observed, with a small 1.65% of cells co-expressing these two markers (Figure 5-7 (B)). The cells were similarly analysed for Rat CD2 and CD8α expression, which similarly identified a small percentage of co-transduced cells (Figure 5-7 (C)).

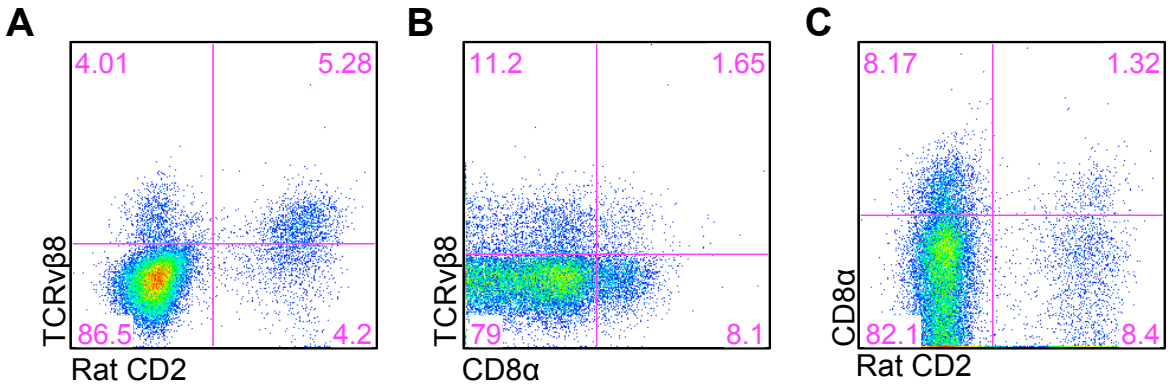

Figure 5-9 Evaluation of Co-transduction of $CD4^+$ T_{REG} Cells. $CD4^+$ T_{REG} cells from healthy control donor T02, were co-transduced with LV encoding the 1E6 WT TCR and CD8-S53N co-receptor. After 72 hours the phenotype of the transduced cells was assessed by staining with (A) α-TCRvβ8 and α-Rat CD2 (B) α-TCRvβ8 and α-CD8α and (C) α-CD8α and α-Rat CD2 antibodies. Data is representative of independent transductions of T_{REG} cells from three donors.

5.3.5. Co-expression of 1E6 TCR and CD8βαS53N can Re-direct the Antigen Specificity of Human CD4$^+$ T_{REG} cells.

Although the percentage of dual 1E6$^+$CD8-S53N$^+$ was low, a peptide stimulation assay was performed and up-regulation of CD69 was examined in four cell populations, characterised by Rat CD2 and CD8-S53N expression (Figure 5-10 (A)). The results from this experiment showed that up-regulation of CD69 in response to ALW peptide was only observed in cells co-expressing Rat CD2 and CD8-S53N (Figure 5-10 (B)). Stimulation with the super agonist RQF peptide resulted in CD69 up-regulation in all Rat CD2$^+$ expressing cells but not in cells expressing CD8-S53N alone. Furthermore, expression of CD8-S53N led to a significant increase in the 1E6 TCRs' response to RQF peptide (Figure 5-10 (B)).

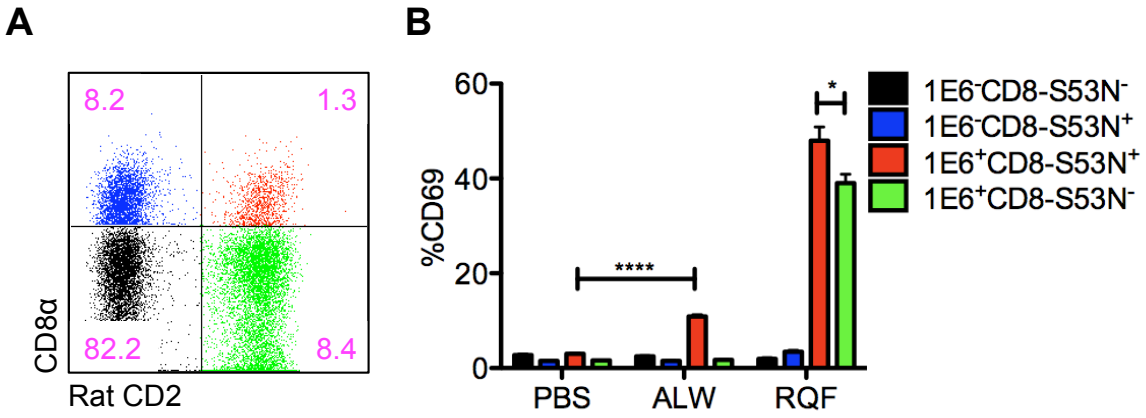

Figure 5-10 Co-expression of CD8-S53N rescues the 1E6$^+$ T_{REG} cells response to WT peptide. CD4$^+$ T_{REG} cells were transduced with LV encoding the WT 1E6 TCR and a second LV encoding CD8-S53N. (A) Transduced cells from donor T02 were stained with antibodies against CD8α and Rat CD2 to assess transduction with both viruses. (B) Transduced cells were incubated with autologous PBMCs pulsed with PBS, ALW or RQF. CD69 was analysed on cell populations shown in (A) and the expression of CD69 in each quadrant was assessed. Data shown is from a single individual and stats calculated from replicates within a single assay. P<0.05 = * and P<0.0001 = **** as calculated by a un-paired Student's t Test.

5.3.6. Combination of CD8-S53N and 1E6 TCR Variants Expression in T_{REG} Cells

The expression of either the 1E6 TCR variants or CD8-S53N co-receptor, led to a significant increase in the response of the 1E6 TCR to RQF peptide. Furthermore, the expression of CD8-S53N enabled signalling via the 1E6 TCR in response to the ALW peptide. It was therefore examined whether co-expression of a 1E6 TCR variant and the CD8-S53N co-receptor could enhance the response of $1E6^+$ T_{REG} cells to ALW peptide. As before, T_{REG} cells were isolated from a healthy control donor, T03, and 48hours post stimulation were co-transduced with CD8-S53N and either 1E6 WT, 1E6 LVL or 1E6 MMu TCR. After 7 days of expansion the cells were then incubated with autologous Violet labelled PBMCs as before. The CD69 expression was then assessed on either the single $1E6^+$ cells or the dual $1E6^+CD8\text{-}S53N^+$ cells (Figure 5-11 (A)). CD69 up-regulation was observed on cells co-expressing either the 1E6 WT, LVL or MMu TCRs with CD8-S53N co-receptor (Figure 5-11 (B)). Interestingly, CD69 expression was significantly increased on cells expressing the 1E6 LVL or 1E6 MMu variant TCRs with CD8-S53N in response to ALW peptide compared to cells expressing the 1E6 WT TCR (mean ±SD for 1E6 WT – 10.9% ±0.69, 1E6 LVL – 17.1% ±2.5 and 1E6 MMu – 18.1 ±2.9). $CD4^+$ T_{REG} cells only expressing the 1E6 TCR up-regulated CD69 in response to RQF but not in response to ALW peptide, irrespective of the 1E6 TCR variant expressed, as seen previously (Figure 5-11 (C)).

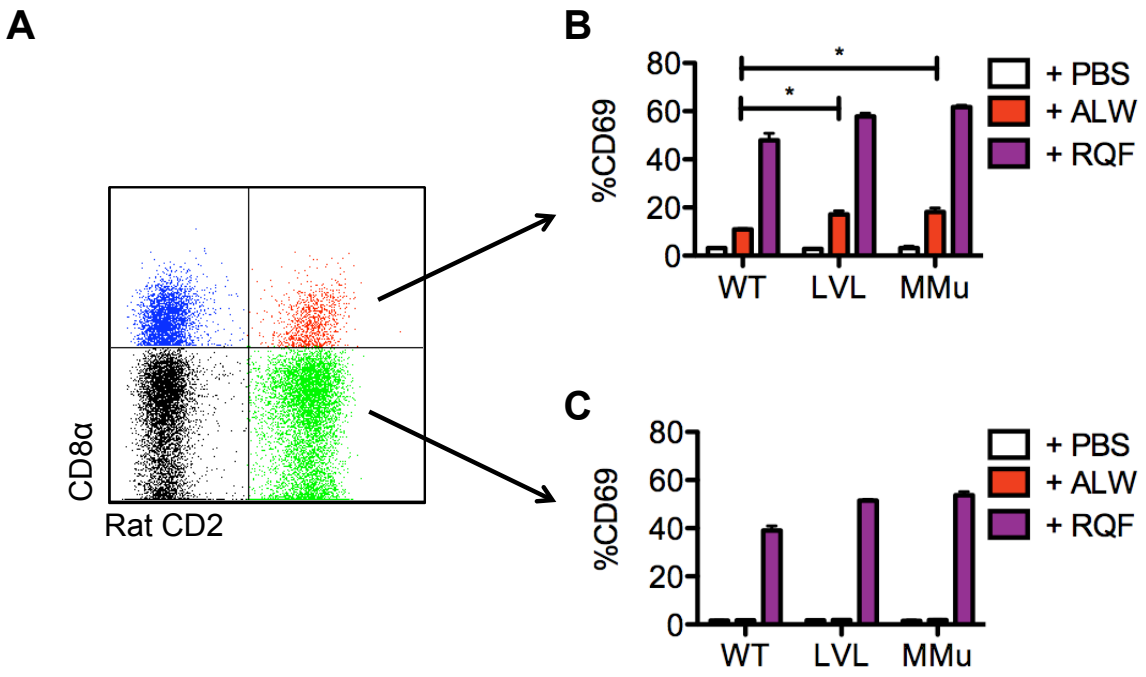

Figure 5-11 Co-transduction of CD8-S53N and 1E6 TCR Variants can Increase the 1E6 TCRs Response to Cognate Peptide. (A) FACS plots show gating of 1E6 and CD8-S53N transduced T_{REG} cells from donor T03. The same gating strategy was used for each 1E6 TCR variant tested. (B) CD69 expression was assessed on dual $1E6^{+}CD8\text{-}S53N^{+}$ T_{REG} cells, as depicted by the arrow. (C) CD69 expression was assessed on single $1E6^{+}$ T_{REG} cells.

5.3.7. Expansion of Dual $1E6^{+}CD8\text{-}S53N^{+}$ T_{REG} cells

From previous results, it appeared that transduction of $CD4^{+}$ T_{REG} cells with CD8-S53N and the 1E6 MMu TCR would be the optimal combination to increase the functional avidity 1E6 TCR-ALW-MHCI interaction. The 1E6 MMu TCR was chosen for these experiments as it had the highest level of expression in $CD4^{+}$ T_{REG} cells. $CD4^{+}$ T_{REG} cells were isolated from a third healthy control donor, T04, and co-transduced with 1E6 MMu and CD8-S53N. As seen previously, the co-transduction of 1E6MMu and CD8-S53N was very low, with only 1.16% cells co-expressing Rat CD2 and CD8-S53N. The transduced T_{REG} cells were expanded in RAPA and after two rounds of expansion, the cells were sorted into three populations of cells: $1E6^{-}$ $CD8\text{-}S53N^{+}$, $1E6^{+}CD8\text{-}S53N^{+}$ and $1E6^{+}CD8\text{-}S53N^{-}$, based on Rat CD2 and CD8 expression (Figure 5-1- (A)). The sorted cells were expanded for one further round of expansion and then stained for expression of CD8 and Rat CD2. The $1E6^{-}CD8$-

S53N$^+$ cells remained Rat CD2$^-$, however lost expression of CD8-S53N, remaining only 67% CD8-S53N$^+$ (Figure 5-12 (B)). Within this population of cells there was also a CD8^{hi} population which is presumed to be contaminating CD8$^+$ T cells, however this contamination was only ~3% of the total population. The sorted 1E6$^+$CD8-S53N$^+$ cells were highly pure for Rat CD2 expression but a quarter of cells lost CD8-S53N expression (Figure 5-12 (C)). Cells that were sorted as 1E6$^+$CD8-S53N$^-$ remained >98% Rat CD2$^+$, however, 30% of these cells co-expressed low levels of CD8-S53N (Figure 5-12 (D)). Since expression of CD8-S53N was lower in this population of cells than for the cells sorted based on CD8-S53N expression, it can be presumed that the FACS gating strategy was not stringent enough to distinguish between CD8-S53N^{low} and CD8-S53N^{hi} cells.

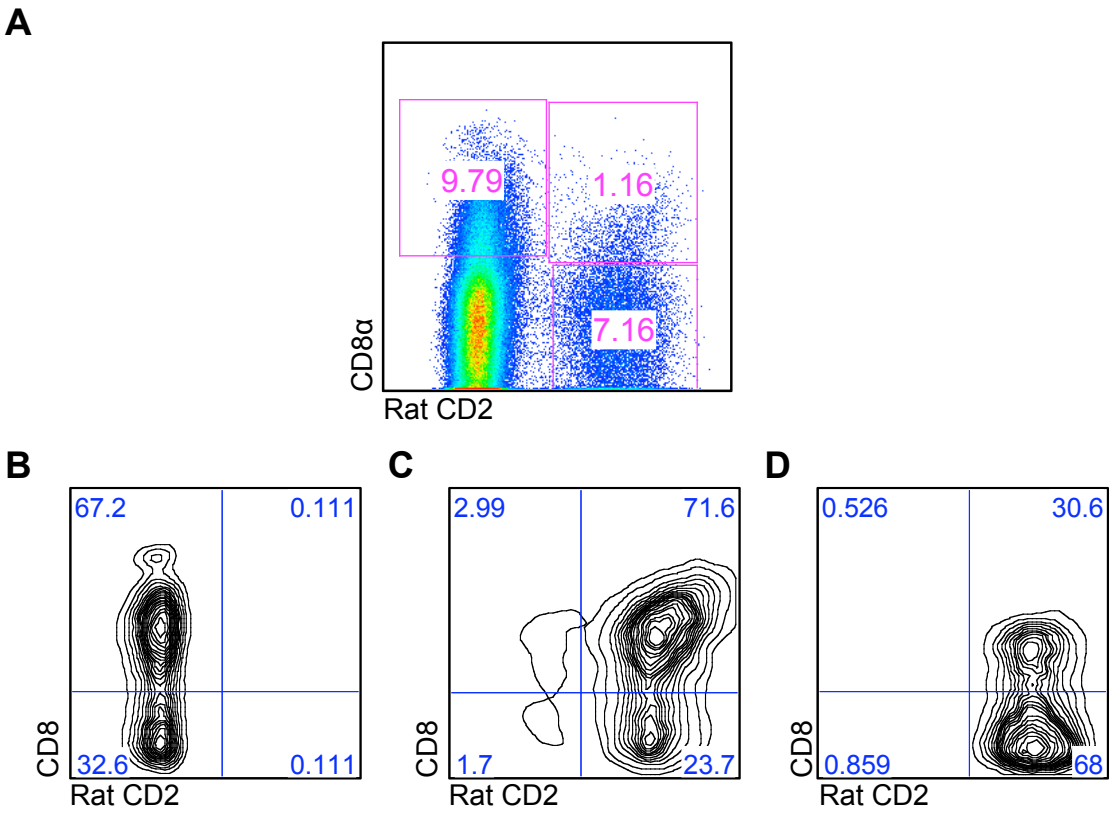

Figure 5-12 Expansion of sorted 1E6$^{+/-}$CD8-S53N$^{+/-}$ from transduced populations of CD4$^+$ T_{REG} cells. CD4$^+$ T_{REG} cells were transduced with 1E6 MMu and CD8-S53N LVs and expanded through two rounds of expansion (A) Transduced cells were sorted into three populations as indicated. The cells were then expanded a third time and stained with antibodies against CD8 and Rat CD2. FACS plots show the phenotype of expanded (B) 1E6$^-$CD8-S53N$^+$ (C) 1E6$^+$CD8-S53N$^+$ and (D) 1E6$^+$CD8-S53N$^-$ T_{REG} cells.

The phenotype of the sorted cells was further assessed by flow cytometry staining of CD3, CD4, CD25 and FoxP3. Due to a limited availability of $1E6^{+}CD8\text{-}S53N^{+}$ cells, these cells were not included in this analysis. However, as all T_{REG} cells from this donor were expanded in the same manner, the phenotypes were predicted to be similar amongst the three populations. Both the $1E6^{-}CD8\text{-}S53N^{+}$ population and the $1E6^{+}CD8\text{-}S53N^{-}$ population were found to be >95% $CD3^{+}CD4^{+}$ (Figure 5-13 (A-B)). A 3% population of contaminating $CD3^{+}CD4^{-}$ cells was observed in the $1E6^{-}CD8\text{-}S53N^{+}$ population, which were also found to be $CD8^{hi}$, identifying these cells as contaminating $CD8^{+}$ T cells seen previously (Figure 5-12 (B)). Furthermore, Intracellular staining for FoxP3 expression identified that both populations of cells were >89% $CD25^{+}FoxP3^{+}$ (Figure 5-13 (C-D)). With confirmation that the sorted populations of cells were >89% $CD4^{+}CD25^{+}FoxP3^{+}$, the cells were cryopreserved to be used in an Ag specific suppression assay performed at a later date.

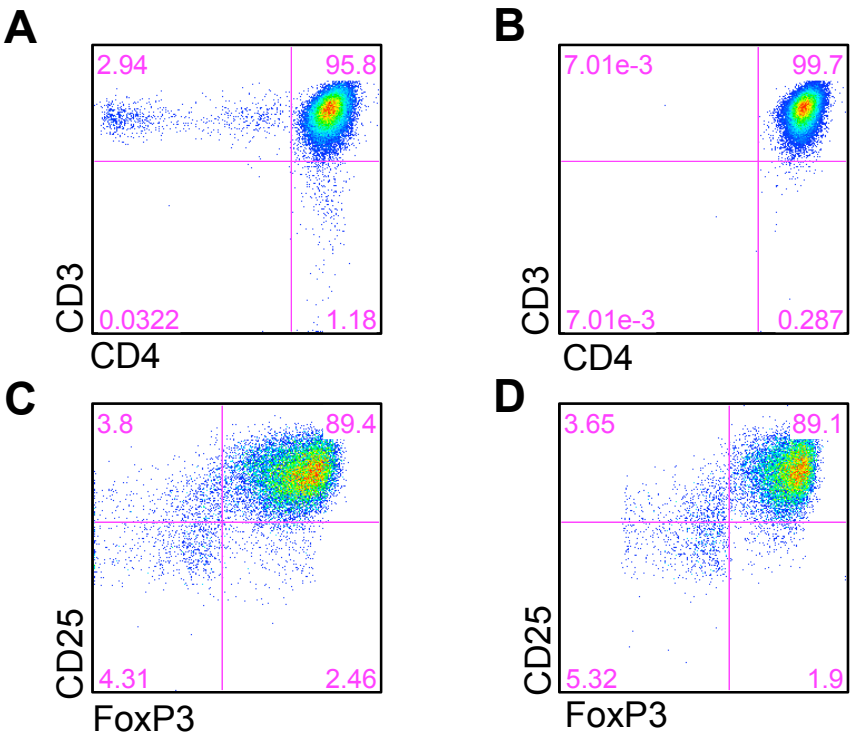

Figure 5-13 Phenotype of Expanded $1E6^{-}CD8\text{-}S53N^{+}$ and $1E6^{+}CD8\text{-}S53N^{-}$ T_{REG} cells. At the end of the third round of expansion, the phenotype of the sorted $1E6^{-}CD8\text{-}S53N^{+}$ and $1E6^{+}CD8\text{-}S53N^{+}$ cells was assessed by flow cytometry. FACS plots show (A) $1E6^{-}CD8\text{-}S53N^{+}$ and (B) $1E6^{+}CD8\text{-}S53N^{-}$ cells stained with αCD3 and αCD4 antibodies. As well as staining for CD25 and FoxP3 expression on (C) $1E6^{-}CD8\text{-}S53N^{+}$ and (D) $1E6^{+}CD8\text{-}S53N^{-}$

5.3.8. Dual CD8-S53N and 1E6 TCR T_{REG} cells can suppress recall responses in an Ag specific manner.

Using a similar suppression assay to that described in chapter 4, the ability of $1E6^{+}CD8\text{-}S53N^{+}$ T_{REG} cells to suppress a recall response when stimulated by the ALW peptide was investigated. Due to a limited availability of dual $1E6^{+}CD8\text{-}S53N^{+}$ T_{REG} cells, a ratio of PBMCs:T_{REG} cells of 10:1 was used. At this lower ratio of T_{REG} cells, $1E6^{+}$ T_{REG} cells stimulated by RQF peptide were previously shown to be effective at suppressing 80% of HA specific $CD4^{+}$ and $CD8^{+}$ T cell proliferation (Figure 4-26). Additionally, the low numbers available of $1E6^{+}CD8\text{-}S53N^{+}$ T_{REG} cells permitted only one replicate (triplicate wells pooled for FACS staining) to be set up per condition. To control for CD8-S53N expression in T_{REG} cells, $1E6^{-}CD8\text{-}S53N^{+}$ T_{REG} cells were included in this suppression assay. The gating strategy for this assay was similar to that used in Chapter 4 in that proliferation of live $CD3^{+}$ T cells was assessed based on FSC-A/SSC-A profile, the exclusion of $7AAD^{+}$ dead cells and the inclusion of $CD3^{+}$ T cells (Figure 5-14 (A)). $1E6^{+}CD8\text{-}S53N^{-}$ and $1E6^{+}CD8\text{-}S53N^{+}$ T_{REG} cells were excluded from analysis of proliferating T cells based on the expression of Rat CD2 (Figure 5-14 (B)). The $CD3^{+}$ T cell population was then separated into $CD4^{+}$ and $CD8^{+}$ T cells for analysis of HA specific proliferation via Violet dye dilution in the subpopulations (Figure 5-14 (C)). In the case of assays including $1E6^{-}CD8\text{-}S53N^{+}$ T_{REG} cells, the initial plan was to exclude these cells on the basis of co-expression of CD8 and CD4. However, as the LV expression of CD8-S53N led to lower expression of CD8 in comparison to natural $CD8^{+}$ T cells, these T_{REG} cells could not be efficiently excluded from the population of HA responding $CD4^{+}$ T cells (Figure 5-14 (C)). Therefore, the effect of $1E6^{-}CD8\text{-}S53N^{+}$ T_{REG} cells on the proliferation of HA specific T cells could not be assessed in this assay.

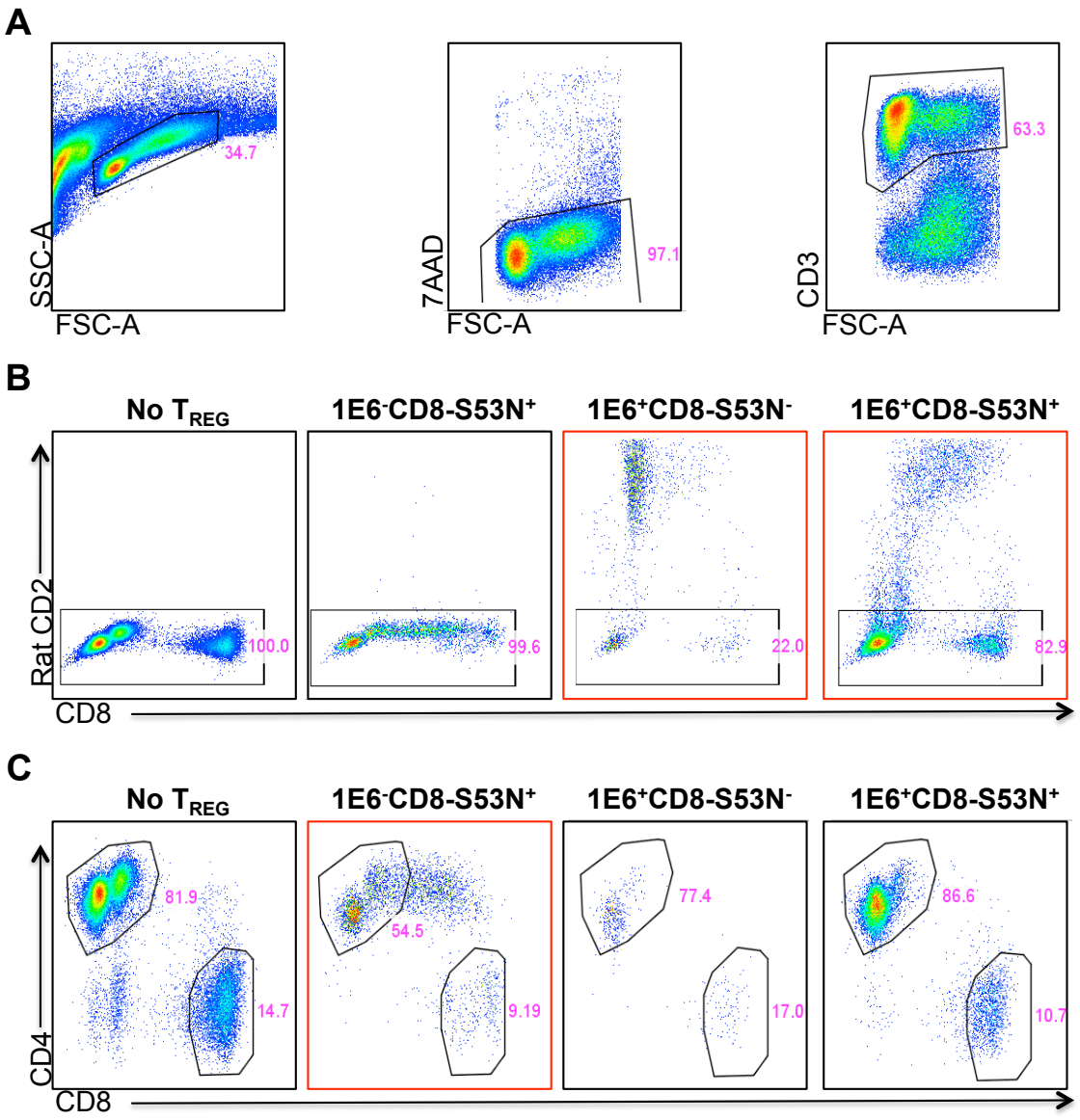

Figure 5-14 Gating Strategy Used for Suppression Assay With 1E6 and CD8-S53N Transduced T_{REG} cells. (A) Lymphocytes were identified based on their FSC-A/SSC-A profile. Dead cells were excluded based on their expression of 7AAD and total T cells were identified by expression of CD3. (B) $1E6^+$ T_{REG} cells were excluded from analysis based on expression of Rat CD2 (highlighted in red). (C) T cells were separated into $CD4^+$ and $CD8^+$ T cell populations to assess Violet dye dilution of each subpopulation. $1E6^-CD8\text{-}S53N^+$ could not be excluded from analysis of $CD4^+$ T cells based on dual expression of CD8 and CD4 alone (highlighted in red).

In the absence of T_{REG} cells, a robust proliferative response by T04 $CD4^+$ T cells to the HA Ag was observed. However, in this donor proliferation was reduced upon addition of ALW peptide, (35% reduction, Figure 5-15 (A&D)), and RQF peptide (28% reduction, Figure 5-15 (A&G)). As observed in all previous suppression assays, the addition of T_{REG} cells in the absence of peptide led to a significant reduction in the proliferation of $CD4^+$ T cells (Figure 5-15 (A-C)). In contrast to previous assays, proliferation was further reduced when $1E6^+CD8\text{-}S53N^-$ T_{REG} cells were stimulated by ALW peptide (Figure 5-15 (B&E)), and this reduction was further enhanced when these cells were stimulated with RQF peptide (Figure 5-15 (B&H)). Stimulation of $1E6^+CD8\text{-}S53N^+$ T_{REG} cells with ALW peptide also resulted in a reduction of $CD4^+$ T cell proliferation compared to that seen when the T_{REG} cells were unstimulated (Figure 5-15 (C&F), which was comparable to stimulation of these cells with RQF peptide (Figure 5-15 (C&I)). A similar pattern in the reduction of proliferation was observed for $CD8^+$ T cells with addition of both T_{REG} cell populations leading to a significant reduction in HA specific proliferation in the absence of peptide (Figure 5-16 (A-C)). A further reduction of proliferation was observed upon addition of either ALW or RQF peptide to stimulate $1E6^+CD8\text{-}S53N^-$ T_{REG} cells. Furthermore, a marked reduction in $CD8^+$ T cell proliferation was observed when $1E6^+$ $CD8\text{-}S53N^+$ T_{REG} cells were stimulated by either ALW or RQF peptide. In all cases, the reduction of proliferation caused by addition of either ALW or RQF to stimulate T_{REG} cells was greater that the addition of peptide in the absence of T_{REG} cells. It is noteworthy that 30% of the $1E6^+CD8\text{-}S53N^-$ T_{REG} cells did in fact express $CD8\text{-}S53N^+$. This may account for the reduction in proliferation of $CD4^+$ and $CD8^+$ T cells upon stimulation of $1E6^+CD8\text{-}S53N^-$ with ALW peptide, which is in contrast to results in Chapter 4. Furthermore, FACS plots represent a single replicate from pooled triplicate wells and therefore further replicates would be required to confirm the results shown.

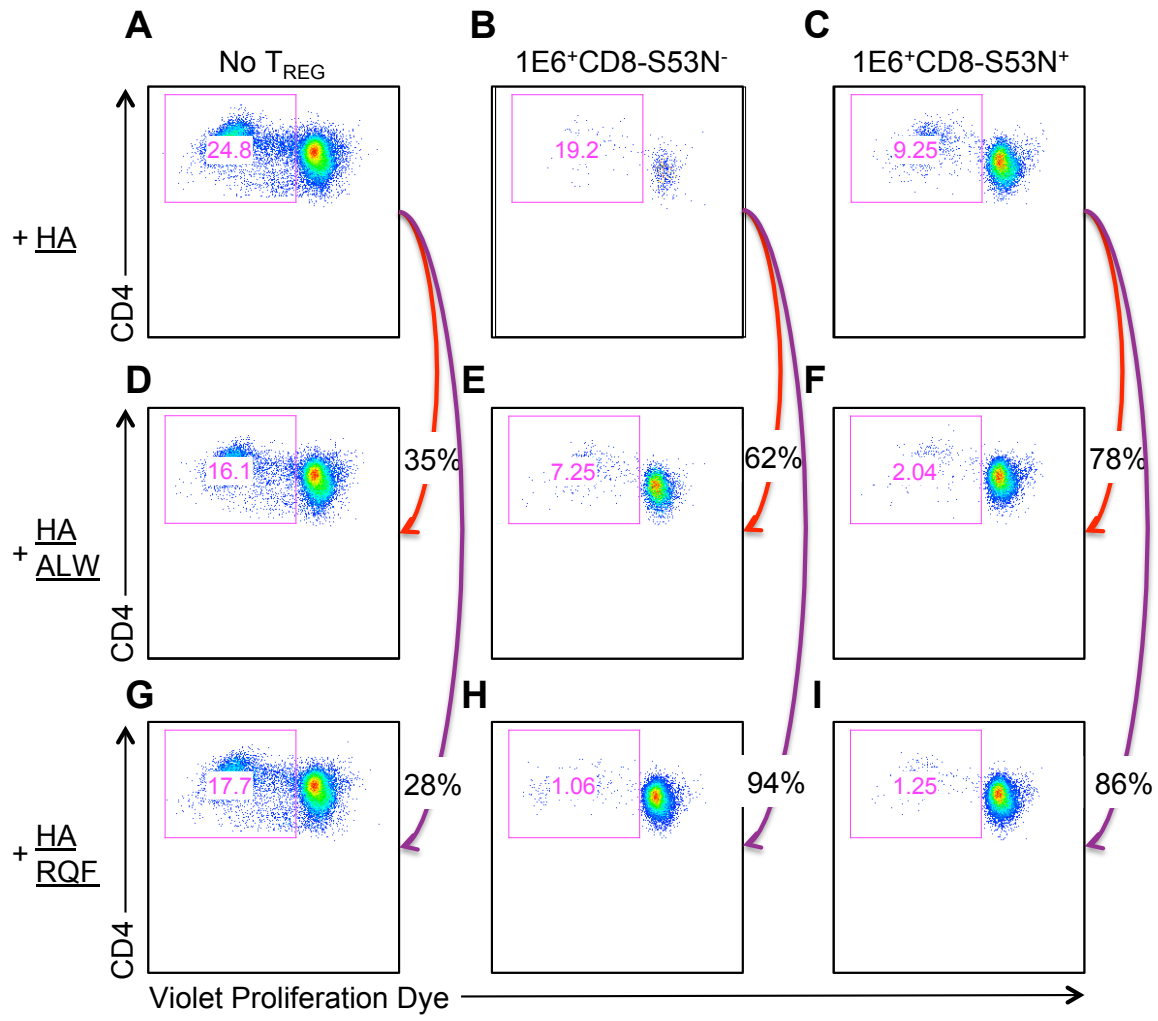

Figure 5-15 FACS plots Showing Reduction of $CD4^+$ T cell Proliferation by Activated $1E6^+$ $CD8\beta\alpha S53N^+$ T_{REG} Cells. Violet labelled PBMCs were incubated with HA +/- ALW peptide or +/- RQF peptide. Representative FACS plots show violet dye dilution of $CD3^+CD4^+$ T cells in the presence of (A) No T_{REG} (B) $1E6^+CD8\text{-}S53N^-$ and (C) $1E6^+CD8\text{-}S53N$. The red arrows indicate the Ag specific suppression on proliferation upon addition of ALW peptide in the absence or presence of the indicated T_{REG} cell population. The purple arrow shows the same but for the addition of RQF peptide.

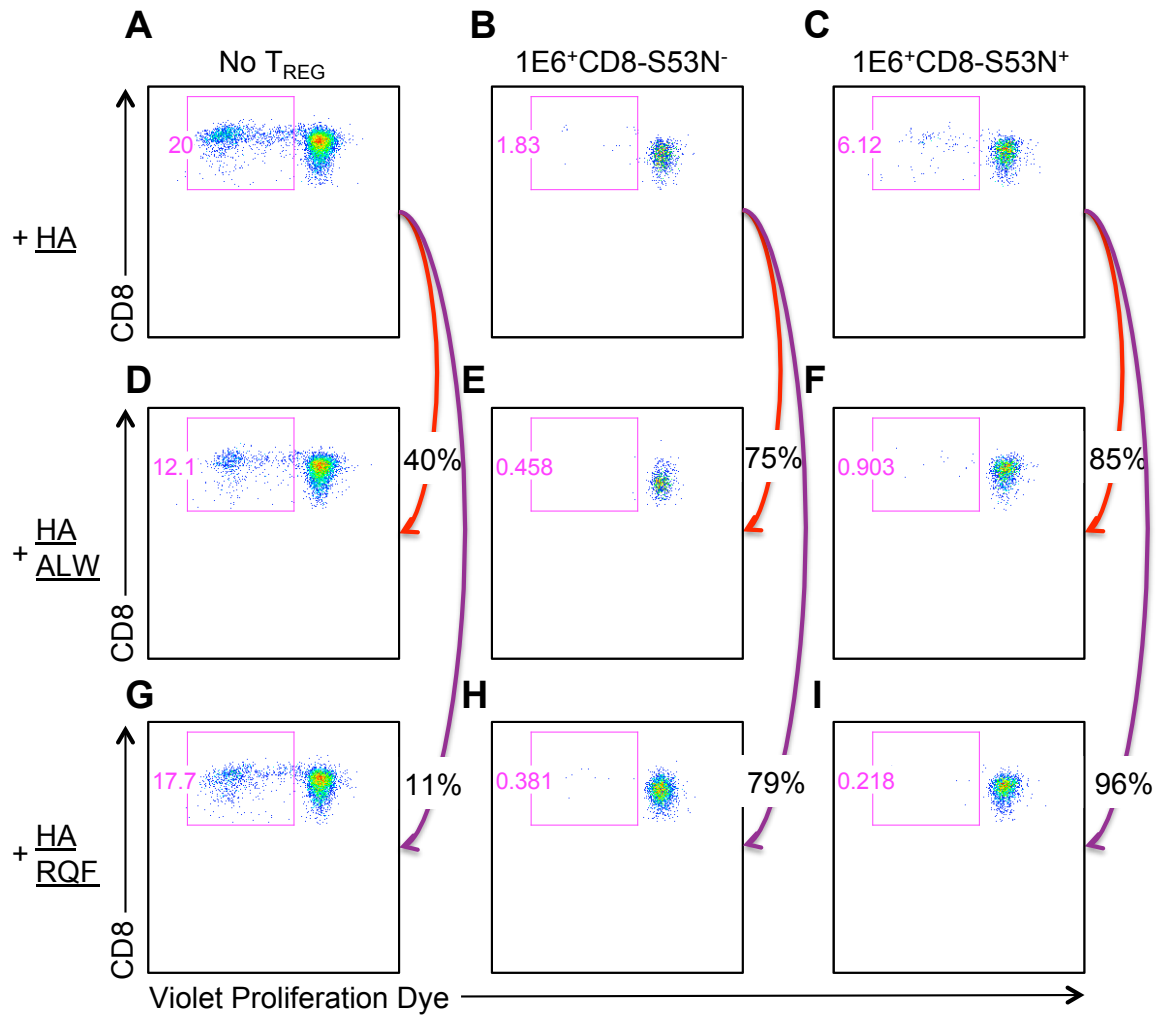

Figure 5-16FACS plots Showing Reduction of $CD8^+$ T cell Proliferation by Activated $1E6^+$ $CD8\beta\alpha S53N^+$ T_{REG} Cells. Violet labelled PBMCs were incubated with HA +/- ALW peptide or +/- RQF peptide. Representative FACS plots show violet dye dilution of $CD3^+CD4^+$ T cells in the presence of (A) No T_{REG} (B) $1E6^+CD8\text{-}S53N^-$ and (C) $1E6^+CD8\text{-}S53N^+$. The red arrows indicate the Ag specific suppression on proliferation upon addition of ALW peptide in the absence or presence of the indicated T_{REG} cell population. The purple arrow shows the same but for the addition of RQF peptide.

Autoreactive T cells in T1D are characterised by secretion of pro-inflammatory cytokines, including IFNγ (Arif et al., 2011; Arif et al., 2004; Cnop et al., 2005). We therefore investigated whether transduced T_{REG} cells, stimulated in an Ag specific manner, could suppress a memory T cells' IFNγ response. As the levels of IFNγ were measured by ELISA and not by FACS, the production of IFNγ by HA responding T cells in the presence of $1E6^{-}CD8\text{-}S53N^{+}$ T_{REG} cells could also be measured in these analyses. The absolute levels of IFNγ (mean ± SD) under each condition tested were as shown (Figure 5-17 (A)). As with proliferation, the addition of T_{REG} cells has a "non Ag specific" suppressive effect on the secretion of IFN-γ by HA stimulated T cells. We therefore compared IFNγ secretion in the presence of T_{REG} cells stimulated by ALW or RQF relative to the level of IFNγ secretion in the presence of un-stimulated T_{REG} cells. Stimulation of both $1E6^{+}CD8\text{-}S53N^{-}$ and $1E6^{+}CD8\text{-}S53N^{+}$ T_{REG} cells with RQF peptide led to a significant reduction in the secretion of IFNγ. However, no such reduction was observed in the absence of T_{REG} cells or in the presence of $1E6^{-}CD8\text{-}S53N^{+}$ T_{REG} cells. Interestingly, stimulation with ALW only led to a reduction of IFNγ in the presence of $1E6^{+}CD8\text{-}S53N^{+}$ T_{REG} cells, and not any other T_{REG} cell population. The mean percentage suppression upon addition of either ALW or RQF peptide was quantified in triplicate wells (Figure 5-17 (B)). These data show that only the stimulation of $1E6^{+}CD8\text{-}S53N^{+}$ T_{REG} cells with ALW peptide led to a marked suppression of IFNγ secretion. In contrast, stimulation with RQF peptide led to a marked suppression of IFNγ secretion by both $1E6^{+}CD8\text{-}S53N^{-}$ T_{REG} cell and $1E6^{+}CD8\text{-}S53N^{-}$ T_{REG} cells.

Taken together, the proliferation and IFNγ secretion data suggest that the co-expression of CD8-S53N and the 1E6 TCR confers suppressive activity to T_{REG} cells in response to the WT ALW peptide. However, due to the severe limitation in T_{REG} cell numbers available, this data is preliminary and a more comprehensive analysis of the suppressive capabilities of $1E6^{+}CD8\text{-}S53N^{+}$ T_{REG} cells in response to ALW peptide is required for confirmation.

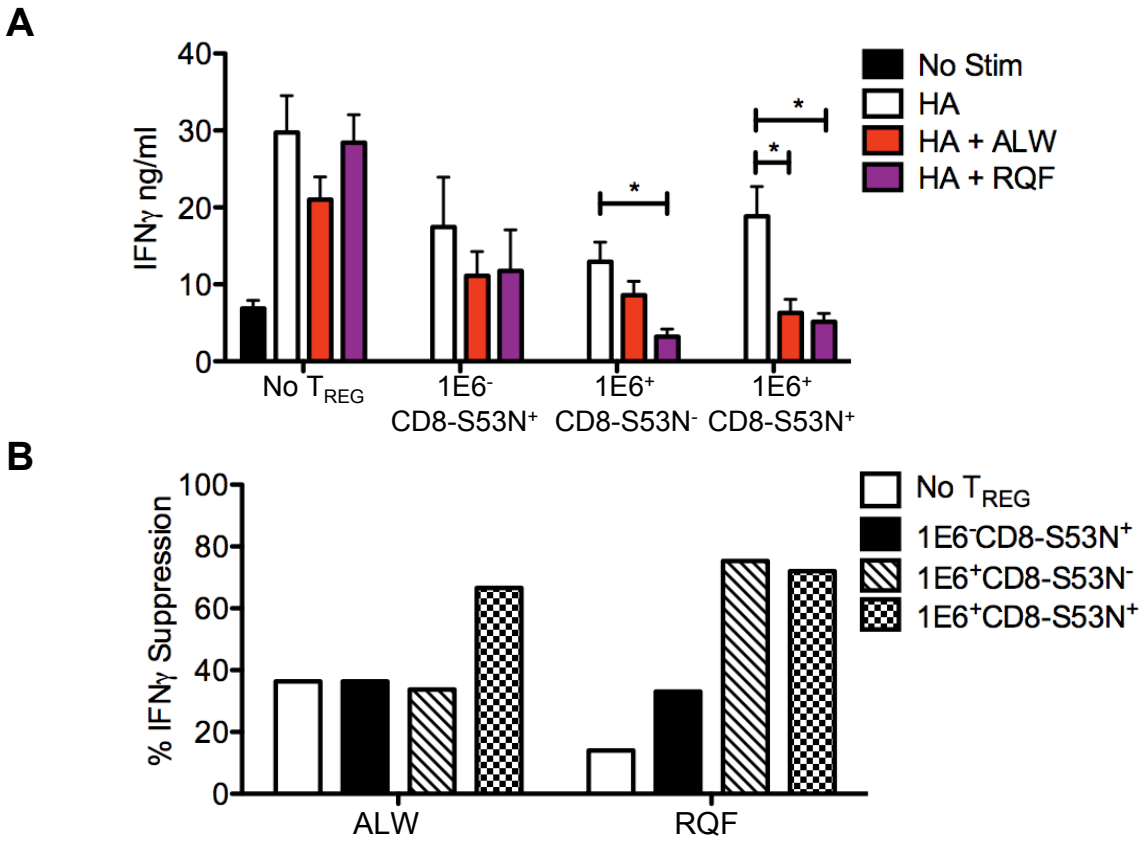

Figure 5-17 Analysis of IFNγ secretion in Culture Supernatants of Ag Specific Suppression Assay. For each well of the Ag specific suppression assay, the secretion of IFNγ was assessed by ELISA. (A) Raw data is plotted for the amount of IFNγ secreted (in ng/ml) per well of the Ag specific suppression assay. An unpaired Student's t test was used to calculate data significance. P * < 0.05. (B) The mean percentage suppression was calculated for the addition of either ALW or RQF peptide in the absence or presence of T_{REG} cell populations.

5.4. Discussion

To date there have been no studies on the transfer of autoreactive MHCI restricted TCRs into human $CD4^+$ T_{REG} cells with a view of re-directing the Ag specificity of these cells. It was determined early on in this thesis that the autoreactive TCRs used in this study had a different pattern of behaviour to the pathogen specific 868 TCR and other pathogen and tumour specific MHCI restricted TCRs used in studies by others (Brusko et al., 2010; Plesa et al., 2012). Results from Chapter 3 and 4 suggest that this altered pattern of behaviour, including weak expression

profiles and a reliance on the CD8 co-receptor, culminated in an unsuitability of these TCRs for the re-direction of the Ag specificity of $CD4^+$ T_{REG} cells. The experiments presented in this chapter sought to identify methods of rescuing the ability of autoreactive MHCI restricted TCRs to re-direct the Ag specificity of human $CD4^+$ T_{REG} cells.

It was first determined whether the responsiveness of the $1E6^+$ T_{REG} cells for WT ALW peptide could be enhanced by increasing the expression and stability of the 1E6 TCR on the surface of transduced cells. To examine this, two 1E6 variant TCRs were obtained, the 1E6 LVL and the 1E6 MMu TCRs. Using the variant TCRs the expression of the 1E6 TCR on the surface of $CD4^+$ T_{REG} cells could be enhanced as measured by an increase in TCR$v\beta8^+$ Rat$CD2^+$ T_{REG} cells. However, this increase of 1E6 TCR on the surface of $CD4^+$ T_{REG} cells was insufficient for the recognition of the WT ALW peptide by transduced T_{REG} cells as measured by CD69 up-regulation in a peptide stimulation assay. The study that first described the LVL modification of TCRs showed that this modification could improve the expression, stability and consequently the function of a number of TCRs (Haga-Friedman et al., 2012). Moreover, the study by *Haga-Friedman et al*, showed an improvement in the function of an MHCI restricted TCR in a $CD4^+$ T_{EFF} cell. However, unlike the 1E6 TCR, the unmodified MHCI restricted TCR used in their study was able to endow a CD4 T_{EFF} cell with Ag specificity, although at a much reduced capacity. It is therefore possible that although an increased expression may lead to an increased avidity interaction between the 1E6 TCR and ALW peptide, this is not sufficient for signalling through this TCR. For this reason, additional methods to improve the stability of the interaction between the 1E6 TCR and ALW-MHCI were investigated.

Consequently, two further LV vectors encoding either the human WT CD8$\alpha\beta$ co-receptor (CD8-WT) or a high affinity variant of this co-receptor (CD8-S53N) were obtained for this study. These vectors were first used to transduce $1E6^+$J76 cells to determine if the LV expression of the full CD8-WT co-receptor could rescue the

response of these cells to the ALW peptide. Indeed, expression of either CD8-WT or CD8-S53N in $1E6^+$J76 cell endowed these cells with the capacity to respond to the ALW peptide. Interestingly, in a dose titration, the magnitude of the response by $1E6^+$J76CD8α cells to ALW peptide was greater than the response by either $1E6^+$CD8-WT^+ J76 cells or $1E6^+$CD8-$S53N^+$ J76 cells. In fact, the response achieved by the $1E6^+$J76CD8α cells was much higher than observed in previous experiments performed in Chapter 3. It was also observed, that the $1E6^+$CD8-$S53N^+$J76 cells exhibited as increased up-regulation of CD69 in response to ALW peptide compared to $1E6^+$CD8-WT^+ J76 cells. Interestingly, in all four $1E6^+$ Jurkat cell lines tested, all cells respond to the RQF peptide but it is clear that $1E6^+$J76CD8α cells respond much better 1E6 and CD8 transduced J76 cells.

In chapter 4 it was shown that the 1E6 TCR could not endow Ag specificity to $CD8^+$ T cells. Therefore, for the progression of this study it was determined that the examination of 1E6 TCR and CD8-S53N co-transduced T_{REG} cells would be the most productive route of study. It was first shown that T_{REG} cells could be transduced with two LV vectors at the same time as evidenced by co-expression of the 1E6 TCR and CD8-S53N. The ability of the $1E6^+$CD8-$S53N^+$ T_{REG} cells to respond to the ALW peptide in a peptide stimulation assay was examined. For the first time in primary T cells, a response to the ALW peptide was observed from 1E6 TCR transduced T_{REG} cells but only when these cells were co-expressing CD8-S53N. Furthermore, the response of $1E6^+$CD8-$S53N^+$ T_{REG} cells was increased when either the 1E6 LVL or 1E6 MMu TCRs were expressed, compared to the 1E6 WT TCR.

With these encouraging results, the ability of the 1E6 TCR, co-expressed with CD8-S53N, to confer suppressive capabilities to $CD4^+$ T_{REG} cells was explored. As the 1E6 MMu TCR had been shown to have the most improved expression and subsequently better functional response compared to the 1E6 WT TCR only 1E6MMu and CD8-S53N co-transduced cells were explored in this setting. From preliminary suppression assay data, it was suggested that these co-expressing

T_{REG} cells were capable of suppressing the proliferation of HA specific $CD4^+$ and $CD8^+$ T cells when ALW peptide was present in the culture. In fact, the reduction in proliferation of the $CD4^+$ and $CD8^+$ T cells observed in the presence of $1E6^+CD8\text{-}S53N^+$ T_{REG} cells was comparable to when the super agonist RQF peptide was present in the culture. As a control for these experiments, the ability of $1E6^+CD8\text{-}S53N^-$ T_{REG} cells to suppress HA proliferative responses when stimulated by the ALW peptide was also assessed. In contrast to what was observed previously, the stimulation of $1E6^+CD8\text{-}S53N^-$ T_{REG} cells with ALW peptide reduced the proliferation of HA specific $CD4^+$ and $CD8^+$ T cells. However, the suppression of $CD4^+$ T cells was lower when $1E6^+CD8\text{-}S53N^-$ T_{REG} cells were stimulated with the ALW peptide compared to the RQF peptide. Characterisation of the $1E6^+CD8\text{-}S53N^-$ T_{REG} cells used in this assay revealed that 30% of these cells were expressing low levels of CD8-S53N, which may account for the ability of these T_{REG} cells to respond to ALW peptide. Due to a limitation in the number of $1E6^+CD8\text{-}S53N^+$ T_{REG} cells this assay could only be performed once, with only one replicate (three replicate wells pooled to form a single replicate) analysed for the reduction of $CD4^+$ and $CD8^+$ T cell proliferation per condition. Thus, the reduction in proliferation observed only points to a trend in the ability of $1E6^+CD8\text{-}S53N^+$ to suppress T cell responses when stimulated by the ALW peptide.

It was encouraging to note that the IFNγ secretion from cultures of PBMCs with HA Ag was significantly reduced in the presence of $1E6^+CD8\text{-}S53N^+$ T_{REG} cells and ALW peptide. This reduction in IFNγ secretion was not observed when ALW peptide was used to stimulate $1E6^+$ or $CD8\text{-}S53N^+$ single positive T_{REG} cells. As previously mentioned, there is strong evidence in human disease and in mouse models that secretion of IFNγ by autoreactive T cells plays a role in β cell death (Arif et al., 2004; Cnop et al., 2005). Therefore, the ability of Ag specific T_{REG} cells to suppress IFNγ secretion could have therapeutic potential, particularly MHCI restricted T_{REG} cells, which have been hypothesised to function at the site of inflammation. The secretion of IL-17 by autoreactive T_H17 cells has also been shown to play a role in inducing β cell death (Arif et al., 2011). It would therefore be

interesting to determine whether Ag specific T_{REG} cells can suppress the production of this cytokine by memory T cells

These experiments will require further repetition, with a number of internal replicates, before the ability of $1E6^{+}CD8\text{-}S53N^{+}$ T_{REG} cell to suppress memory T cell proliferative responses can be fully examined. A more appropriate control for these experiments perhaps would be to split the initial T_{REG} cell population prior to LV transduction, into cells to be transduced with 1E6 LV only and cells to be co-transduced with 1E6 and CD8-S53N LV. This would prevent any contamination of the single $1E6^{+}$ T_{REG} cells with cells co-expressing low levels of CD8-S53N. Unfortunately, a low initial yield of T_{REG} cells was obtained from donor T04, and with the aim to improve cell yields, all cells were co-transduced and sorted into the described populations at the end of two rounds of expansion.

To improve upon these data, the suppression assays involving dual 1E6 TCR and CD8-S53N expressing T_{REG} cells need to be repeated. Unfortunately, a low level of co-transduction of these two receptors, which was < 2% of T_{REG} cells, led to a very low yield of these cells at the end of three rounds of expansion. In the data shown two co-transductions were performed using T_{REG} cells from two different donors and in every instance the co-expression of 1E6 and CD8-S53N was below 2%. The co-transduction and expansion of T_{REG} cells was repeated in a third donor, however after three rounds of expansion there were not enough $1E6^{+}CD8\text{-}S53N^{+}$ T_{REG} cells to perform a meaningful suppression assay. Unfortunately, time constraints prevented further optimisation of the co-transduction of T_{REG} cells, however there are methods that could be pursued in future experiments. It may be optimal to transduce T_{REG} cells with the 1E6 LV during the first round of expansion, select the transduced cells and then transduced the selected cells with CD8-S53N during the second round of expansion. Dual expressing $1E6^{+}CD8\text{-}S53N^{+}$ T_{REG} cells could then be selected at the end of the second or third round of expansion. Alternatively, as $1E6^{+}CD8\text{-}S53N^{+}$ T_{REG} cells can recognise ALW-pMHCI, as shown in peptide stimulation assays, it may be possible to expand these cells with ALW

peptide pulsed APCs. This would permit the selective outgrowth of cells that can respond to the ALW peptide, which would negate the requirement for cell sorting of transduced cells. Additionally, it has been shown that $CD4^+$ T_{EFF} cells can be transduced with a multi-cistronic vector encoding TCR genes and the CD8αβ co-receptor genes, which results in co-expression of both TCR and CD8αβ (Xue et al., 2013). Thus an LV vector that encodes both the 1E6 TCR and CD8-S53N from a single promoter could be engineered. However, it would need to be fully determined that all genes could be expressed to the same extent. In Jurkat cells, it was observed that the Rat CD2 and CD8α genes were compromised when co-expressed on the same LV vector. However, in the transduction of primary T cells, the expression of the Rat CD2 gene is expressed to the same extent, and sometimes greater than the TCRvβ genes. Therefore, engineering a single vector may be worth pursuing.

Due to low cell numbers, the effect of CD8-S53N expression in a T_{REG} cell was not explored in this study. In theory, the expression of the CD8-S53N co-receptor in T_{REG} cells should be safe as the co-receptor can not interact with MHCII and therefore will only aid the interaction between the introduced 1E6 TCR and pMHCI. The study by *Xue et al* transferred an MHCI restricted TCR into $CD4^+$ and $CD8^+$ T cells and identified that the re-directed $CD8^+$ T cells had a 10-fold higher avidity than the $CD4^+$ T cells (Xue et al., 2013). Interestingly, the co-expression of the TCR and CD8αβ co-receptor genes in $CD4^+$ T cells enabled a functional avidity that rivalled the response exhibited by $CD8^+$ T cells. Importantly, the expression of the CD8αβ co-receptor did not alter the cytokine profile of the re-directed $CD4^+$ T cells, therefore suggesting that CD8 expression in a $CD4^+$ T cell cannot alter $CD4^+$ T cell function. However, the safety of CD8-S53N expression in a $CD4^+$ T_{REG} cell will need to be fully elucidated in future studies. For example, the full cytokine range that expanded dual $1E6^+CD8\text{-}S53N^+$ T_{REG} cells secrete upon recognition of the ALW peptide could be examined. However, it would not be possible to compare cytokine secretion profile of single $1E6^+$ T_{REG} cells activated with ALW peptide to examine any differences in cytokine secretion.

In summary, the results presented here elude to the ability of a high affinity CD8-S53N to rescue the capacity of low affinity autoreactive MHCI restricted TCRs in re-directing the Ag specificity of human $CD4^+$ T_{REG} cells. Although the suppression assay data is preliminary, the ability of $1E6^+CD8\text{-}S53N^+$ T_{REG} cells to up-regulate CD69 in response to ALW peptide is encouraging. This therefore, is the first study, which has successfully engineered $CD4^+$ T_{REG} cells to recognise an MHCI restricted autoimmune disease relevant Ag through LV gene transfer.

6. Final Discussion

Adoptive T_{REG} cell therapy, using polyclonal T_{REG} cells, is currently being investigated as a therapy to halt T1D progression (Marek-Trzonkowska et al., 2012; Putnam et al., 2009). Evidence from mouse models however, indicates that T_{REG} cells with islet Ag specificity would be more efficacious at controlling T1D pathogenesis than polyclonal T_{REG} cells (Tang et al., 2004; Tarbell et al., 2007). Although, human islet Ag specific T_{REG} cells can be identified or generated from naïve T cells, the number and stability of expanded Ag specific T_{REG} cell may not be suitable for human therapy (Brusko et al., 2008). To generate a large number of Ag specific T_{REG} cells, this study utilised LV vectors to transfer TCR genes into human T_{REG} cells. In this manner, large number of T_{REG} cells can be generated to any desired Ag specificity. Moreover, this study investigated the gene transfer of MHCI restricted TCRs to T_{REG} cells. This was based on the hypothesis that MHCI engineered T_{REG} cells would be more effective than MHCII restricted T_{REG} cells at entering autoimmune tissues and eliciting Ag specific suppression.

In agreement with previous studies, MHCI restricted TCRs can re-direct the Ag specificity of T_{REG} cells to Ags that have a high affinity interaction with the introduced TCR (Brusko et al., 2010; Plesa et al., 2012). It had previously been suggested that naturally occurring low affinity MHCI restricted TCRs could re-direct the Ag specificity of T_{REG} cells but not $CD4^+$ T_{EFF} cells (Plesa et al., 2012). However, this study identified that this is not the case for all low affinity MHCI restricted TCRs. The 1E6 TCR was unable to re-direct the Ag specificity of $CD8^+$ T cells, T_{EFF} cells or T_{REG} cells in response to its cognate PPI peptide. High expression of CD8α in TCR$\alpha\beta^-$ Jurkat cell lines could rescue the low affinity interaction between the 1E6 TCR and ALW-MHCI. However, a reduced capacity to compete with endogenous TCRs in primary cells led to a reduced expression of the 1E6 TCR that was insufficient to re-direct the Ag specificity of $CD8^+$ or $CD4^+$ T cells including T_{REG} cells. Although increasing the expression of the 1E6 TCR in T_{REG}

cells was unable to rescue the TCRs response to ALW peptide, the co-expression of a high affinity CD8$\alpha\beta$ co-receptor, CD8-S53N was capable of rescuing the interaction between 1E6 TCR and ALW-MHCI This suggests, that very low affinity islet Ag specific MHCI restricted TCRs require further modifications or co-receptor engagement to confer potent suppressive capabilities to T_{REG} cells. This study therefore extends the knowledge base of the clinical applicability of using autoreactive MHCI restricted TCRs to confer potent suppressive capabilities to human T_{REG} cells

6.1. Enhancing MHCI Restricted TCRs for Gene Therapy

TCR signalling upon engagement of pMHC molecules is reliant on a number of different factors, such as the affinity of the interaction, the engagement of co-receptors and clustering of TCRs at the immunological synapse. It has previously been shown in $CD8^+$ T cells that the higher the affinity a TCR has for its pMHC the less reliant this interaction is on the CD8 co-receptor (Holler and Kranz, 2003). Therefore, methods to increase the affinity of the TCR-pMHC interaction or the introduction of the CD8 co-receptor in $CD4^+$ T cells could theoretically enhance MHCI restricted TCR function. Furthermore, an increase in the expression of the introduced TCR on the cell surface would seek to promote T cell activation by improving TCR serial triggering and overall functional TCR-pMHC interaction avidity.

It has been observed in this study and by others that certain so called “weak TCRs” are ill equipped to outcompete endogenous TCRs for cell surface expression (Heemskerk et al., 2007; Stauss et al., 2007). This leads to a reduced expression of the introduced TCR on the transduced T cell surface as observed with the autoreactive 1E6 and 1D7 TCRs. As previously mentioned, the productive hit rate model of T cell activation relies on serial triggering of multiple TCRs by pMHC complexes (Valitutti et al., 1995). This is aided by formation of the immunological synapse, which is a clustering of TCRs with adhesion, co-stimulatory and signalling

molecules (Grakoui et al., 1999). T cell activation occurs when the overall avidity of the TCR-pMHC interactions reaches the threshold for productive T cell signalling. Therefore, a greater number of low affinity TCR-pMHC interactions would need to take place than the number of high affinity interactions. Subsequently, the low expression of the 1E6 TCR, even in the context of $CD8^+$ T cells, prevents the avidity threshold being reached in response to the ALW peptide. In contrast, the low expression of the TCR had no effect on the ability of the TCR to signal in response to the super agonist peptide, irrespective of the cell context. Due to time constraints, it was not examined whether the increased expression of the 1E6 TCR on $CD8^+$ T cells could promote signalling in response to ALW peptide. The examination of this would fully test the hypothesis that the inability of the 1E6 TCR to signal in $CD8^+$ T cells was due to low expression.

An additional method to improve introduced TCR surface expression not explored in this study, is the removal of endogenous TCRs. In addition to removing competition for expression with the endogenous TCR this would improve the safety of TCR gene transfer by forgoing the potential of TCR mis-pairing and formation of neo-reactivities (van Loenen et al., 2010). Zinc finger nucleases, delivered by viral vectors, can be used to disrupt endogenous TCRα and TCRβ chains at the genetic level, imprinting cells and their progeny as TCR$\alpha\beta^-$. Cells can then be reconstituted with any TCR of choice by LV gene transfer. TCR gene transfer into TCR-edited T cells yields improved expression of the introduced TCR as well as superior Ag recognition in comparison to TCR gene transfer in non-edited T cells (Provasi et al., 2012). However, the process of TCR gene editing in this study was arduous and contained four viral transduction steps, including two rounds or cell sorting and αCD3/CD28 stimulation, before re-directed T cells were produced. For TCR gene therapy of low numbers of $CD4^+$ T_{REG} cells, this method would reduce the recovery of transduced cells.

An alternative route to stabilising low affinity TCR pMHC interactions by co-expressing the CD8-WT or CD8-S53N co-receptor in $CD4^+$ T_{REG} cells would be to

isolate or engineer MHCI restricted TCRs with a high enough affinity for pMHC so that the CD8 co-receptor is dispensable. It has been suggested that the natural TCR affinity range of TCRs is between 1μM and 100μM (Thomas et al., 2011). To put this into context of the current study, the 1E6 TCR has a reported affinity below this range of 278μM and the 868 TCR above this range of 0.08μM. Unlike B cells, T cells do not undergo affinity maturation *in vivo*. To circumvent this, techniques have been developed to engineer T cells that have a higher affinity for Ag *in vitro*. TCRs with mutations in the CDR3 regions of TCRα and TCRβ chains can be screened for improved peptide binding using yeast phage display and has been used to identify TCRs with supra-physiological affinities for pMHC (Li et al., 2005). For example, Varela-Rohena and colleagues engineered the 868 TCR to possess a 360-fold increase in affinity, which conferred a superior control over the spread of HIV *in vivo* compared to WT 868 TCR (Varela-Rohena et al., 2008). However, others have identified that the affinity maturation of TCRs can lead to reduced Ag specificity or impact the ability to recognise low concentrations of pMHC (Thomas et al., 2011; Zhao et al., 2007). The study by *Thomas et al* identified that the increased TCR affinity, caused by an increased TCR-pMHC dwell time, resulted in reduced serial TCR triggering. Therefore, increasing the affinity of the TCR-pMHC interaction did not affect its functional avidity. Additionally, the safety of affinity matured TCRs was called into question in 2013, when the transfer of cells expressing an affinity matured TCR, exhibited toxicity due to cross reactivity that was not predicted during *in vitro* testing (Cameron et al., 2013; Linette et al., 2013). It may however be useful to test affinity-matured 1E6 TCRs in their ability to re-direct the specificity of T_{REG} cells, whilst taking into consideration the described results and safety concerns.

6.2. Identification of autoreactive MHCI Restricted TCRs

From the data in this study, it can be determined that the successful re-direction of $CD4^+$ T_{REG} cells using autoreactive MHCI restricted TCRs is reliant on the TCRs high affinity for self-Ag. However, the process of negative selection makes the

identifications of TCRs with these properties in circulation highly unlikely. As previously mentioned, during negative selection T cells that possess TCRs that react too strongly against self-pMHC complexes are deleted from the T cell repertoire. In this way, only T cells that have a high affinity for Ags not presented in the thymus will exit into the periphery. It is however possible to look out-with the normal TCR repertoire for $CD8^+$ T cells with a high affinity for self Ag by turning to patients with defects in negative selection or alternatively, HLA transgenic mice.

Autoimmune polyendocrinopathy syndrome type 1 (APS-1) is a rare disorder characterised by a spectrum of organ specific autoimmune diseases, including T1D. In the course of identifying the genetic determinants of this disease, a loss of function mutation was found in the gene now known as the autoimmune regulator (*Aire*) gene (Nagamine et al., 1997). Since the discovery of the *Aire* gene, its critical role in negative selection has been revealed. To first identify the function of this gene, a seminal study by *Liston et al* employed the 3A9 TCR transgenic mouse model whose T cells possess high avidity TCRs for an Ag of hen egg lysosome (HEL) (Liston et al., 2003). When the HEL gene is expressed under the control of the rat insulin promoter (RIP), the majority of high avidity HEL specific T cells are deleted in the thymus through negative selection. However, when these mice were crossed with $Aire^{-/-}$ mice, a complete failure in the thymic deletion of HEL specific T cells was observed. It is now known that *Aire* is expressed in the thymic medullary epithelial cells and is responsible for the promiscuous gene expression of thousands of tissue specific Ags that are presented to developing T cells in the thymus. Consequently, the failure of negative selection in these patients makes it plausible that they harbour high affinity T cells specific for T1D associated Ags such as insulin. It is possible, that T cells with a high affinity for self Ag may be deleted or rendered anergic in the periphery by mechanisms of peripheral tolerance. However, these patients may provide a window of opportunity for the isolation of autoreactive T cells with a higher than normal affinity for self-Ag. Potentially, MHCI multimer staining could be used to identify T1D associated Ag specific $CD8^+$ T cells from these patients. Additionally, modified MHCI multimers

that do not interact with human CD8 could be used to identify high affinity CD8 independent TCRs (Choi et al., 2003). Cloning of the TCR genes from these T cells could potentially yield MHCI restricted TCRs more potent at re-directing the Ag specificity of T_{REG} cells than those used in the current study.

HLA transgenic mice are engineered to express human MHC molecules and are a useful tool in the generation of high affinity, human Ag specific TCRs (Theobald et al., 1995). One such example is the HHD transgenic mouse, which expresses a human-mouse hybrid HLA-A2*01 MHCI molecule (Pascolo et al., 1997). The hybrid transgene consists of human β_2M-HLA-A2*01 peptide binding domains, α1 and α2, coupled to the murine α3 domain from H-2D^b MHCI molecules. Importantly, these mice lack murine MHCI molecules due to a disruption in the murine *B_2M* and *H-2D^b* genes ensuring the full CD8$^+$ TCR repertoire is restricted to human HLA-A2*01. This model has previously been used by the group of Steve Rosenberg to identify high avidity TCRs specific for the human melanoma associated Ag gp100:64-162 (Johnson et al., 2009). This epitope differs from the murine epitope at a single amino acid, and immunisation of HHD mice with the human gp100 enabled the identification of a high avidity TCR. T cells transduced with the resulting high avidity murine TCR were shown to be capable of mediating cancer regression in 19% of melanoma patients. The main caveat of using murine derived TCRs in TCR gene therapy however is the development of a xenogeneic immune response against the adoptively transferred T cells. In a follow up study, a humoral response to murine TCRs was identified in 25% of patients treated with T cells expressing murine TCRs. It was promising to note however that the development of antibodies against the transferred T cells was not consistent with reduced T cell persistence or responsiveness to therapy (Davis et al., 2010). In 2010, transgenic HHD mice that possess a diverse human TCR repertoire were described (Li et al., 2010). Using yeast artificial chromosomes the full human TCRα and TCRβ loci were expressed in HHD mice deficient of murine TCRα and TCRβ genes. Importantly, the immunisation of these mice with a human melanoma derived Ag enabled the identification of high avidity Ag specific CD8$^+$ T cells. Therefore, identification of PPI

specific $CD8^+$ T cells in these mice would permit the identification of fully human TCRs from HHD mice.

Human insulin protein has only 82% and 79% shared sequence homology with murine insulin 1 and insulin 2 protein respectively. Furthermore, the human PPI_{15-24} ALW epitope used in this study has a 70% disparity to the same murine epitope of insulin 2, the murine form of insulin that is predominately expressed in the thymus (Chentoufi and Polychronakos, 2002). Theoretically, the $CD8^+$ T cells of HHD mice would not be negatively selected on the basis of a high affinity TCR for the HLA-A2*01 restricted ALW epitope and would be permitted entry into circulation. Based on this rationale, HHD mice could be immunised with the human ALW epitope of PPI to identify high affinity HLA-A2*01 restricted TCRs. Of particular interest in this study, high affinity TCRs that are CD8 independent may be indentified for the re-direction of human $CD4^+$ T_{REG} cells. The α3 domain of MHCI molecules, which in the HHD mouse is murine derived, provides the CD8 binding domain. Importantly, there is no cross binding between the murine α3 domains with human CD8$\alpha\beta$ (Purbhoo et al., 2001). In theory, fully human HLA-A2 multimers could be used to identify Ag specific $CD8^+$ T cells that are independent of human CD8.

The described immunisations have been carried out in collaboration with Farzin Farzenah (Rayne institute, Kings college London). In total, one mouse was immunised with PBS and three with a high dose of ALW peptide following a previously described immunisation protocol that enables combined and synergistic triggering of DCs to promote potent $CD8^+$ T cell responses (Wells et al., 2008). One of the three immunised mice yielded a potent $CD8^+$ T cell response to the ALW peptide as evidenced by ALW HLA-A2 multimer staining (Figure 6-1). Splenocytes and lymphocytes from all mice have since been isolated and cryopreserved for future analysis. It is hypothesised that HLA-A2 ALW $multimer^+$ cells could be isolated and expanded. It would then be possible to isolate TCR genes following the previously described 5' RACE clonotyping protocol from ALW specific $CD8^+$ T cells.

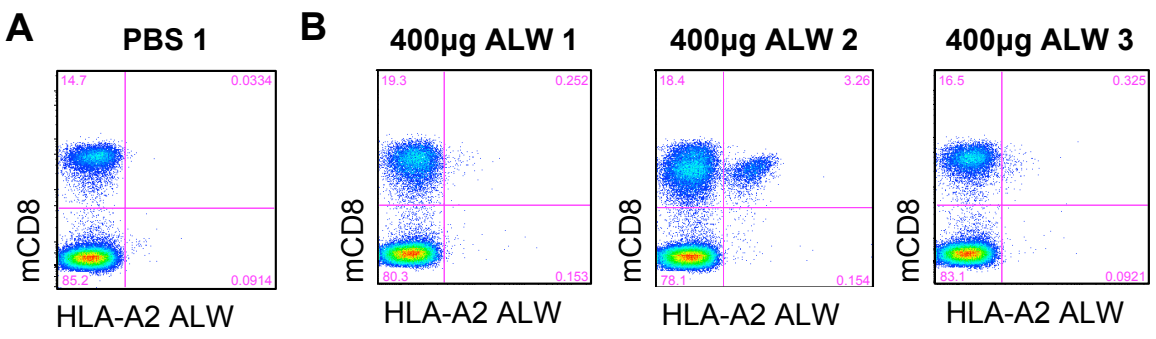

Figure 6-1 Immunisation of HHD Mice with PPI_{15-24} Epitope. HHD mice were immunised as shown and after 3 weeks tail blood was stained with an HLA-A2 ALW multimer followed by α murine CD8 and CD3 antibodies. Staining with CD3 was used to analyse multimer staining of total $CD3^+$ T cells and FACS plots show staining of (A) a single mouse immunised with PBS and (B) mice immunised with a 400µg dose of ALW peptide.

6.3. Future of MHCI Restricted TCR Gene Therapy in $CD4^+$ T_{REG} cells

On target toxicity exhibited by TCR gene modified T cells is one of the biggest concerns of the adoptive transfer of T cell therapy of $CD4^+$ or $CD8^+$ T cells. Another concern in TCR gene therapy is mis-pairing of endogenous and introduced TCRα and TCRβ genes, which may form harmful neo-reactivity's (Bendle et al., 2010; Rosenberg, 2010). Although, TCR mis-pairing has been observed in pre-clinical mouse models, mis-pairing of TCRα and TCRβ genes has so far not been observed in the human setting. Theoretically, on target toxicity does not pose as big a threat using gene modified T_{REG} cells, as these cells should not exhibit the deleterious effects observed from TCR engineered T_{EFF} cells. Therefore, the main safety concern of TCR gene therapy of $CD4^+$ T_{REG} cells is the unintended transfer of an autoreactive MHCI restricted TCR into T_{EFF} or $CD8^+$ T cells. The unwitting transfer of autoreactive T_{EFF} cells into patients with T1D could exacerbate and worsen disease state, particularly for MHCI restricted TCRs as they could directly recognise and target MHCI expressing islets. Therefore, it is imperative that only the purest populations of T_{REG} cells are used for this therapy. As discussed, there is no unique marker that selectively identifies T_{REG} cells. However isolation of

$CD4^+CD25^+CD127^{lo}CD45RA^-$ may reduce the risk of T_{EFF} cell contamination (Hoffmann et al., 2006; Miyara et al., 2009; Putnam et al., 2009). Towards the end of this study, RAPA was used in T_{REG} cell expansion with a view of promoting the sole expansion of T_{REG} cells (Battaglia et al., 2006). Although, a direct comparison between RAPA expanded and non-RAPA expanded T_{REG} cells would be required to confirm any benefits provided from the inclusion of RAPA.

The stability and plasticity of $CD4^+$ T_{REG} cells in mice has also been called into question, particularly at sites of inflammation. Genetic fate mapping studies of $FoxP3^+$ T_{REG} cells identified an accumulation of "$exFoxp3^+$" T_{REG} cells in inflammatory sites (Zhou et al., 2009). However, it has since been suggested that these "$exFoxp3^+$" T_{REG} cells arise from non-committed, transiently activated cells and that true suppressive T_{REG} cells do not undergo reprogramming under inflammatory conditions (Miyao et al., 2012). FoxP3 stability is maintained epigenetically by the DNA demethylation of the TSDR region within the $FoxP3^+$ gene. It would therefore be prudent to determine the demethylation status of the TSDR region within LV transduced and expanded populations of T_{REG} cells. Prior to adoptive T_{REG} cell therapy with cells expressing MHCI restricted TCRs, it would be crucial to determine the stability of these cells.

It has been suggested that one mechanism of Ag specific T_{REG} cell suppression is the direct killing of T_{EFF} cells and APCs in a granzyme or perforin dependent manner (Cao et al., 2007; Gondek et al., 2005; Grossman et al., 2004). Both adaptive and activated $CD4^+CD25^+$ human T_{REG} cells have been shown to kill autologous target cells (Grossman et al., 2004). Naturally occurring islet Ag specific, IL-10 secreting T_{REG} cells have also been found to selectively kill APCs expressing specific pMHCII (Tree et al., 2010). Therefore, the expression of MHCI restricted islet Ag specific TCRs in T_{REG} cells could potentially endow these cells with the ability to recognise and kill islets. A more recent study has suggested that expanded $CD4^+CD25^+$ and not $CD4^+CD25^+CD127^{low}$ T_{REG} cells display cytotoxicity activity post stimulation with recombinant bispecific antibodies (Koristka et al.,

2014). However, this has not been assessed using Ag specific T_{REG} cells. Due to low cell numbers of dual $1E6^{+}CD8\text{-}S53N^{+}$ T_{REG} cells, it was not possible to test in this study the ability of these cells to kill in response to pMHCI. In future experiments, cytotoxicity assays can be performed using Ag pulsed HLA-A2 expressing K562 cells as surrogate islets and $1E6^{+}CD8\text{-}S53N^{+}$ T_{REG} cells as effector T cells. Transduced T_{REG} cells that had been incubated with surrogate islet cells could also be stained for expression of CD107, a marker of degranulation. These experiments could shed light on the cytotoxicity capabilities and safety of these cells.

In this study, Rat CD2 was used solely as a marker in the LV expression cassette to evaluate *in vitro* transduction efficiencies of cells. LV expression vectors can encode up to 10kb and therefore additional genes can be encoded to promote the safety of TCR gene therapy. To reinforce the regulatory function of transferred T_{REG} cell, the FoxP3 gene could be expressed in the LV vector and co-transferred with the TCR genes. In mice, the transfer of a FoxP3 gene to islet Ag specific T_{EFF} cells endowed the T_{EFF} cells with suppressive capabilities, enabling them to reverse diabetes in mice with recent onset disease (Jaeckel et al., 2005). To alleviate safety concerns of the transfer of TCR gene re-directed T_{REG} cells, suicide genes could be included in the LV expression vector in the place of the Rat CD2 marker gene. The B cell marker CD20 has been investigated as a suitable suicide and marker gene as its expression can be detected by flow cytometry, and cells expressing this gene can be killed after exposure to the therapeutic αCD20 monoclonal antibody, Rituximab (Introna et al., 2000). More recently, the use of a compact marker/suicide gene comprised of CD34 and CD20 epitopes (RQR8) has been described. The use of the RQR8 gene enabled selection of transduced cells via the GMP approved CliniMACS CD34 system (Miltenyi Biotec, Germany) as well as retaining the Rituximab binding site of CD20 (Philip et al., 2014). However, treatment with Rituximab to remove transduced T cells would also deplete B cells for up to 6 months or more post treatment. Another potential suicide gene is caspase 9, a late stage component of the apoptotic pathway. Expression of an

inducible caspase 9 can cause apoptosis of transduced cells upon exposure to a chemical inducer of dimerisation (CID) (Straathof et al., 2005). Patients undergoing haploidentical stem cell transplantation were co-transfused with donor T cells transduced to express the inducible caspase 9 suicide gene (Di Stasi et al., 2011). Upon the first signs of GVHD development, patients were treated with the CID, AP1903, and 90% of donor T cells were eliminated within 30 minutes of CID administration. Importantly, the treatment with CID AP1903 has no serious side effects.

6.4. Suitability of TCR Gene Modified T_{REG} Cells as a Treatment for T1D.

The safety of the adoptive transfer of gene modified T cells has already been demonstrated in a number of clinical trials (Johnson et al., 2009; Robbins et al., 2011). However, to date, the use of gene modified T cells has not been explored as a treatment for autoimmune diseases. The incidence of T1D is rising in the western world and a therapy that aims to prevent or treat the causes of T1D, as opposed to the effects is greatly required. The transfer of polyclonal autologous T_{REG} cells have already shown to be safe and well tolerated in patients with newly onset T1D (Marek-Trzonkowska et al., 2014; Marek-Trzonkowska et al., 2012). The integration of a transgene into the host genome poses the risk of insersertional mutagenesis. However to date there have been no reports of this using cells modified by LV vectors. Furthermore, the improved safety in viral base gene therapy and availability of GMP grade facilities for isolation, transduction expansion of T cells will significantly aid the transition of TCR gene therapy of T_{REG} cells in T1D.

Pan immunosuppressive agents, such as cyclosporine have been shown to ameliorate the effects of T1D. However the number of side effects associated with this treatment is an unacceptable risk in T1D, particularly in the young (Silverstein et al., 1988). This has paved the way for the identification of more specific forms of

immunosuppression in T1D that pose little widespread effects. The use of islet Ag specific T_{REG} cell therapy should fit this remit as in theory their suppressive effects will be exhibited within the islets and pancreatic draining lymph nodes. However, it is possible that the administration of islet Ag specific T_{REG} cells could have pan immunosuppressive effects. As was observed in suppression assays in chapters 4 and 5, the presence of T_{REG} cells could reduce proliferation of HA responding $CD4^+$ and $CD8^+$ T cells, although this reduction significantly increased when T_{REG} cells were activated by their specific peptide. Additionally, evidence from mouse models suggests that lower numbers of Ag specific T_{REG} cells, compared to polyclonal T_{REG} cells would be required for disease prevention and reversal (Tang et al., 2004). Therefore, the adoptive transfer of reduced numbers of T_{REG} cells may prevent any pan immunosuppressive effects. Furthermore, the use of MHCI restricted TCR expressing T_{REG} cells should aid the homing of T_{REG} cells specifically to the islets, which would also reduce pan immunosuppressive effects. Therefore, the ability of adoptive transferred islet Ag specific T_{REG} cells to home to the pancreatic draining lymph nodes and islets is essential to this therapy. It has been observed previously that expanded T_{REG} cells retain expression of the lymph node homing markers CD62L and CCR7 (Hoffmann et al., 2004). It would be important for this study to determine expression of these markers post LV transduction and expansion. Expression of the chemokine receptor CXCR3 is important for homing of autoreactive T cells to the islet via the chemokine CXCL10 (van Halteren et al., 2005). Human T_{REG} cells expressing CXCR3 that migrate in response to CXCL10 have been identified (Hoerning et al., 2011). Therefore, it would be interesting to assess the level of CXCR3 on the LV transduced and expanded T_{REG} cells used in this study and whether these cells can migrate in response to CXCL10 using chemotaxis assays. This would give an idea of whether T_{REG} cells generated in this study would be able to home to the islets as expected.

A major consideration in using islet Ag specific T_{REG} cells as a treatment for T1D is the timing at which they are administered. A Josilin Medalist study identified that residual β cell function and turnover can be found in patients with T1D, even when

disease duration was over 50 years (Keenan et al., 2010). Moreover, studies of pancreata from patients with T1D suggest that upon T1D diagnosis, 50% of β cell mass remains (Coppieters et al., 2012). Importantly, in a mouse model of autoimmune diabetes, only the transfer of islet Ag specific T_{REG} cells, and not polyclonal T_{REG} cells could reverse disease (Tang et al., 2004). These studies would suggest that immunotherapies aimed at preserving β cell mass could reverse the requirements of exogenous insulin in patients who have previously been diagnosed with T1D. Therefore, adoptive islet Ag specific T_{REG} cell therapy need not be selectively targeted to patients recently diagnosed with T1D. Risk of T1D development in first-degree relatives can be predicted by assessing genetic predisposition, presence and number of islet autoantibodies and metabolic testing (Achenbach et al., 2005). If the use of islet-Ag specific T_{REG} cells is deemed safe with little side effects then these could also be used as an interventional therapy. Alternatively, T_{REG} cells for adoptive transfer could be generated from high-risk individuals, cryopreserved and used as a therapy upon initial diagnosis.

A concern with using adoptive T_{REG} cell therapy to treat patients with T1D is that the inherent genetic defects that predispose patients to disease would reduce the efficacy of this therapy. A number of the genetic polymorphisms associated with T1D predisposition, particularly in the IL-2 signalling axis, are implicated in defective T_{REG} cell function (Dendrou et al., 2009; Garg et al., 2012; Long et al., 2010). The low dose IL-2/RAPA trial, although showing no clinical improvement in subjects with T1D, did identify an improvement in the overall T_{REG} cell responsiveness to IL-2 that persisted for at least a year post treatment (Long et al., 2012). Therefore, expansion of T_{REG} cell in high dose IL-2, which is the protocol currently used, may overcome defects in IL-2 responsiveness. Moreover, reduced IL-2 responsiveness in patients with T1D is associated with a loss of FoxP3 maintenance (Garg et al., 2012; Long et al., 2010). However, this defect could be overcome by including *FoxP3* in the LV construct when generating Ag specific T_{REG} cells by LV TCR gene transfer. Studies have also shown that the T_{EFF} cells from patients with T1D are more resistant to T_{REG} cell mediated suppression (Lawson et

al., 2008; Schneider et al., 2008). It has been suggested however that the resistance of T_{EFF} cells to be suppressed may be overcome by increasing T_{REG} cell numbers. The use of Ag specific T_{REG} cells may also aid in overcoming T_{EFF} cell resistance to suppression. To fully test this hypothesis however, the methods described in this study using T_{REG} cells from healthy controls will need to be explored fully in T_{REG} cells from patients with T1D. The generation of Ag specific T_{REG} cells from patients will allow for full examination of the ability of these cells to suppress autologous T_{EFF} cell Ag specific responses.

The final step for this study would be to assess the efficacy of 1E6CD8-S53N T_{REG} cells in a humanised mouse model of autoimmune diabetes. In a HLADR4 expressing immunodeficient NOD-*scid* mouse, the transfer of human islet autoreactive $CD4^+$ T cells could selectively infiltrate murine islets and selectively destroy β cell (Viehmann Milam et al., 2014). As the 1E6 TCR is HLA-A2 restricted, then previously described NOD-*scid.HLA.A2.1* mice would serve as a useful humanised mouse model to test the efficacy of 1E6CD8-$S53N^+$ T_{REG} cells. Additionally, due to the disparity between murine and human preproinsulin genes, transgenic mice expressing the human insulin gene would be required (Selden et al., 1986). Using these transgenic mice, the ability of the 1E6 $CD8^+$ T cell clone to transfer diabetes could first be assessed. Adoptive transfer studies of $1E6^+$CD8-$S53N^+$ T_{REG} cells, or other MHCI restricted expressing T_{REG} cells, could be performed to assess the ability of these cells to prevent or reverse autoimmune diabetes. Alternatively, humanised mouse models have been used to examine the efficacy of human alloantigen specific T_{REG} cells in mediating tolerance to an allogeneic human skin graft (Sagoo et al., 2011). A similar approach could be taken to examine the efficacy of human TCR gene modified T_{REG} cells in suppressing the rejection of transplants of human islet cells. In a NOD-*scid* mouse model, it would be possible to transplant human HLA-A2/$DR4^+$ islets under the kidney capsule of these mice. To promote autoreactivity to the transplanted islets, HLA-A2 restricted $CD8^+$ or HLA-DR4 restricted $CD4^+$ T cells that are specific for islet Ags could be adoptively transferred. The efficacy of MHCI islet Ag specific

T_{REG} cells at protecting the human islets from T cell mediated destruction could then be efficiently assessed in adoptive T_{REG} studies.

6.5. Concluding Remarks

This study provided the first evidence that the specificity of $CD4^+$ T_{REG} cells can be re-directed towards a MHCI restricted autoimmune disease relevant Ag. The 1E6 TCR is so far the best-described autoreactive MHCI restricted TCR available. Due to its low affinity for self-peptide this TCR required the co-expression of a high affinity CD8 co-receptor for the successful re-direction of $CD4^+$ T_{REG} specificity. However, the safety of CD8 co-receptor transfer into human $CD4^+$ T_{REG} cells requires further assessment. For the successful progression of this therapy it may be prudent to identify further MHCI restricted TCR that would be suitable for transfer into human T_{REG} cells without additional enhancement. This can be approached by using either HLA transgenic mice or cells from patients with APS-1. This would enable further investigations into the feasibility of autoreactive MHCI restricted TCR gene modified T_{REG} cells as a therapy in the treatment of T1D.

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